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(71) Applicant: SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH [US/US]; 1275 York Avenue, New York, NY 10021 (US).		Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(72) Inventors: SCHIAVO, Giampietro; Via L. Cadorna, 16, I-35121 Abano T. Padua (IT). ROTHMAN, James, E.; 402 East 64th Street, New York, NY 10021 (US). SÖLLNER, Thomas, H.; 83 Stratford Avenue, White Plains, NY 10605 (US).			
(74) Agent: SONNENFELD, Kenneth, H.; Morgan & Finnegan, LLP., 345 Park Avenue, New York, NY 10154 (US).			

(54) Title: METHODS AND COMPOSITIONS FOR MODULATING VESICLE RELEASE

(57) Abstract

This invention provides methods and compositions useful for modulating synaptic transmission. The compositions of this invention are complexes comprised of various combinations of proteins, such as tagmin, β -SNAP, NSF, α -SNAP, and SNAREs involved in docking and fusion of synaptic vesicles to membranes. In addition, this invention relates to complexes of tagmin and special phospholipids (phosphatidyl inositols) which are a component of membranes involved in the secretory process. These complexes are useful for identifying other substances which may modulate synaptic transmission.

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- 1 -

METHODS AND COMPOSITION FOR MODULATING VESICLE RELEASE

This application claims priority to pending United States provisional patent application Serial No. 60/030,867 filed November 12, 1996 and pending United States provisional patent application 60/008,596 filed December 13, 1995, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

This invention relates to methods and compositions useful for modulating the release of vesicular contents from cells. Generally, this invention relates to modulating the process whereby intracellular vesicles fuse with the cell membrane to cause the release of the vesicular contents. More particularly, this invention relates to modulating the releases of synaptic vesicles from neurons. In addition, this invention relates to compositions and methods useful for modulating secretion of substances produced in cells. This invention also relates to methods and compositions useful for screening substances which modulate vesicle release.

BACKGROUND OF THE INVENTION

Vesicle release from cells, particularly neurons, is a complex process requiring the proper function of various components of the cytoskeleton and soluble proteins. Appropriate ionic flux and response to such flux is also an important aspect of vesicle release. These molecular components and fluxes function together to control the release of neurotransmitter from neurons by providing for calcium mediated fusion of neurotransmitter containing vesicles to the synaptic membrane of the presynaptic neuron. Molecular aspects of synaptic vesicle

- 2 -

release have recently been reviewed in Burns et al., Cell 83:187-194 (1995) and Südhof, Nature, 375:645-653 (1995). Exocytosis of transport vesicles has been described in other cells and organisms including yeast. Wilson D.W. et al., Nature, 339:355-359 (1989).

5 Various proteins have been reported to be involved in synaptic vesicle release including: N-ethylmaleimide-sensitive fusion protein (NSF)⁵, synaptotagmin (p65 or tagmin)^{2,8}, soluble NSF attachment proteins (alpha, beta and gamma SNAP) and SNAP receptors (SNAREs)³. Although these proteins have been associated with release of synaptic vesicles their roles and mechanisms of action are still unclear.

10 Synaptic vesicle release is believed to result from at least a three step process following the appropriate electrical stimulus. In the first process, docking, the synaptic vesicle is mobilized from a pool of filled vesicles and binds to the presynaptic plasma membrane at active sites. After docking, a slow ATP hydrolysis dependent step termed "vesicle priming" occurs.

15 This priming process makes exocytotic synaptic vesicles competent for the third step, fast calcium dependent fusion. The membranes of the synaptic vesicle and plasma membrane fuse in the last step releasing the vesicle contents into the synaptic cleft. Unfortunately, little has been known about these three steps and the specific proteins involved affording little opportunity for intervention with specific drugs. As recently reviewed, "The proteins responsible for docking vesicles at the membrane are not yet clear, but may include the GTP-

20 binding protein Rab3 as well as UNC-18 or SNARE proteins (or both) ..." Burns et al., Cell, 83:187-194, 187 (1995).

25 A number of proteins have been implicated in the rapid (msec) calcium-controlled release of transmitters at nerve endings^{1,2}, including the general fusion proteins α -SNAP (soluble NSF attachment protein)^{3,4} and NSF (NEM-

- 3 -

° sensitive fusion protein)^{3,5,6}, the synaptic SNARE (SNAP receptor)^{3,7}, and the calcium-binding protein synaptotagmin², which likely functions as calcium sensor in exocytosis⁸. In addition, an isoform of α -SNAP termed β -SNAP (83% identical to α -SNAP) is highly expressed in
5 brain⁹; no special role for β -SNAP has yet been reported. It has been unclear how, or if, these separate threads of understanding are entwined to account for synaptic transmission.

Recently, Mikoshiba and coworkers²⁰ have found
10 that the C2B domain of tagmin binds polyphosphoinositols, specifically InsP₄, InsP₅ and InsP₆, but not InsP₃.

SUMMARY OF THE INVENTION

15 The present invention relates to methods and compositions useful for identifying substances capable of modulating synaptic transmission. In particular, a protein complex has been identified which is involved in docking of synaptic vesicles to synaptic membranes in
connection with synaptic transmission. This complex is
20 comprised of three proteins β -SNAP, tagmin, and NSF and the complex, according to this invention, may be used as a target for substances such as drugs which modulate synaptic transmission. Intermediate complexes comprising
 β -SNAP and tagmin bind NSF and may also be a target for drugs which modulate synaptic transmission. Another intermediate comprising tagmin, β -SNAP, and NSF binds
25 α -SNAP to form a quaternary complex which is capable of binding to SNAREs (syntaxin, VAMP and SNAP25). An aspect of this invention therefore is a cell free protein complex comprising a protein comprising amino acid sequences of β -SNAP sufficient to bind to tagmin and a protein comprising an amino acid sequence of tagmin capable of binding β -SNAP. Another aspect of this invention is a cell free complex comprising a protein comprising amino acid
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- 4 -

o sequences of β -SNAP sufficient to bind to tagmin, an amino acid sequence of tagmin capable of binding β -SNAP and NSF protein. Still another complex is the tagmin/ β -SNAP/NSF complex further comprising α -SNAP and, optionally the SNAREs. Complexes comprising tagmin and
5 phosphatidylinositol bisphosphate ("PI_ns-P₂") and phosphatidylinositol trisphosphate ("PI_ns-P₃") are another aspect of this invention.

Another embodiment of this invention is the use of the formation of the cell free β -SNAP/tagmin, β -SNAP/tagmin/NSF, or β -SNAP/tagmin/NSF/ α -SNAP or β -SNAP/tagmin/NSF/ α -SNAP/SNARE, or any of the above cited 10 complexes also comprising PI_ns-P₂, or PI_ns-P₃, complexes as an assay for the detection of drugs which modulate synaptic transmission. Accordingly, this invention also relates to assay kits comprising the components to conduct such assays including β -SNAP, tagmin and optionally NSF, α -SNAP, SNARE proteins, PI_ns-P₂, PI_ns-P₃, and various reagents for detecting the presence of a protein in the 15 complex. These components should be present in an amount sufficient to detect complex formation in the absence of a test substance.
20

The method of identifying substances capable of inhibiting synaptic transmission comprises combining an amino acid sequence of β -SNAP capable of binding tagmin and a β -SNAP binding protein comprising an amino acid sequence of tagmin with a test substance under conditions which allow for β -SNAP and the β -SNAP binding protein to form a complex and then detecting the formation of β -SNAP/ β -SNAP binding protein complexes. Optionally, the 25 formation of a triple complex further comprising NSF or a quaternary complex further comprising α -SNAP may be assayed in the presence of a test substance. In addition the complex comprising β -SNAP/tagmin/NSF/ α -SNAP and at least one SNARE protein may also be used in this 30 invention. In either assay, substances which are
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- 5 -

° identified which reduce or inhibit the formation of any of the β -SNAP/tagmin, β -SNAP/tagmin/NSF, β -SNAP/tagmin/NSF/ α -SNAP, β -SNAP/tagmin/NSF/ α -SNAP/SNARE, tagmin/PIns-P₂, or tagmin/PIns-P₃, complexes may be expected to similarly reduce or inhibit synaptic transmission. Conversely, 5 substances capable of increasing complex formation would be expected to facilitate synaptic transmission.

Because different isoforms of tagmin are localized in different parts of the nervous system 10 including different brain regions (see, Ullrich et al., Neuron, 13:1281-1291 (1994)) another embodiment of this invention includes assays for detecting substances which modulate synaptic transmission which are specific for neurons or regions of the nervous system based on the 15 presence of a particular tagmin isoform. Accordingly, in this embodiment of the invention, assays are conducted using specific isoforms of tagmin which are specific for the region of the nervous system for which synaptic transmission is desired to be modified.

20 The binding of the β -SNAP/tagmin/NSF complex to α -SNAP and SNAREs are additional sites provided by this invention for modulating docking and thereby synaptic transmission.

This invention also provides methods of 25 modulating synaptic transmission by modulating formation of either the β -SNAP/tagmin, β -SNAP/tagmin/NSF, β -SNAP/ tagmin/NSF/ α -SNAP, or β -SNAP/tagmin/NSF/ α -SNAP/SNARE complex or tagmin with any of the above cited complexes 30 with PIns-P₂, or PIns-P₃. By providing substances which are specific for certain isoforms of tagmin, this invention also provides highly specific means of modulating synaptic transmission in vivo. Polyphosphoinositols are a class of compounds (PIns-P₂, PIns-P₃ and analogues) provided by this 35 invention which may be used to modulate docking and synaptic transmission.

- 6 -

The synaptic vesicle membrane protein synaptotagmin (tagmin) is essential for fast, calcium-dependent, neurotransmitter release. The present invention has identified a connection between the observations that polyphosphoinositides are necessary for exocytosis and the finding that the C2B domain of tagmin I binds phosphatidylinositol-4,5-bisphosphate (PIns-4,5-P₂), its isomer phosphatidylinositol-3,4-bisphosphate (PIns-3,4-P₂) and phosphatidylinositol-3,4,5-trisphosphate (PIns-3,4,5-P₃). Calcium ions switch the specificity of this binding from PIns-P₃ (at calcium concentrations found in resting nerve terminals) to PIns-P₂ (at concentration of calcium required for transmitter release). The present invention teaches that tagmin operates as a bimodal calcium sensor, switching bound lipids during exocytosis, which is useful in treatment and maintenance of various physiological processes and disorders associated therewith, such as long-term memory and learning. The present invention can also be used to treat various neurological disorders comprising increasing or decreasing the intracellular calcium levels in a patient.

The ability to specifically modulate synaptic transmission by identifying drugs which are specific for various isoforms of tagmin provides a means for specifically treating various neurological disorders associated with improper synaptic transmission; i.e. anatomically different areas of the nervous system.

Antibodies which are specific for the various complexes are also provided by this invention which may be used for either diagnostic or therapeutic purposes.

An object of this invention is to provide compositions and methods for identifying substances useful for decreasing or inhibiting functional docking or priming of vesicles to plasma membranes.

Another object of this invention is to provide compositions and methods useful for inhibiting synaptic

- 7 -

° transmission mediated through docking of synaptic vesicles to SNARES.

Another object of this invention is to provide substances useful for treating various neurological or psychiatric disorders.

5 Yet another object of the present invention is to treat various neurological and psychiatric disorders by modulating neurotransmitter release by manipulating the intracellular calcium levels in a recipient with such a disorder.

10 Another object of this invention is to provide assay systems and kits useful for identifying such substances.

This invention casts new light on synaptotagmin by affording the recognition that this protein is not only a calcium sensor but also a specialized v-SNARE. Like other v-SNAREs³, synaptotagmin is localized to a transport vesicle, binds to a t-SNARE (syntaxin)¹⁴, is a specialized SNAP receptor (binds β -SNAP, but not α -SNAP), enters a docking and fusion particle, and is released when NSF hydrolyzes ATP. The docking and fusion complexes provided by this invention could serve to link, directly or indirectly, the general process of membrane fusion to calcium entry by attaching a specialized fusion protein (β -SNAP) to a v-SNARE that is also a calcium sensor (synaptotagmin). The polyphosphoinositols InsP₄, InsP₅, and InsP₆ are known to block transmitter release and also block the assembly of the particle by preventing β -SNAP from binding to synaptotagmin.

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BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Schematic representation of the formation of protein complexes involved in vesicle docking.

- 8 -

° **Fig. 2.** Binding specificity of α -SNAP and β -SNAP for SNAREs and to tagmin. A) Schematic representation of synaptotagmin I and its fragments (hatched boxes, C2 domains; solid box, transmembrane region). B) Specific binding of β -SNAP to tagmin and to its carboxy-terminal part and the subsequent recruitment of NSF (left panel). C) α -SNAP and β -SNAP binding to the SNARE complex.

10 **Fig. 3.** Requirements for the assembly of the specialized docking and fusion particle. Immobilized GST-tagmin/ β -SNAP/NSF complexes and GST were incubated with bovine brain detergent extracts in absence or presence of α -SNAP or β -SNAP. Bound syntaxin 1A and 1B (upper panel), SNAP-25 (middle panel) and VAMP (lower panel) were analyzed by 15 immunodecoration of Western blots.

20 **Fig. 4.** Effect of polyphosphoinositols (IHPS) on the binding of β -SNAP to tagmin. Immobilized GST-tagmin was 25 incubated with β -SNAP in the presence of different IHPS at increasing concentrations. The results were expressed as percentage of the amount of β -SNAP bound to tagmin in the absence of IHPS. (closed squares, InsP_6 ; open triangles, InsP_5 ; closed circle, InsP_4 ; open squares, InsP_3) and represented the average of three to five independent experiments.

30 **Fig. 5.** Mg^{2+} ATP dependent disassembly of the specialized docking and fusion particle. Immobilized complexes were treated under conditions that allow Mg^{2+} ATP dependent SNARE complex disassembly. This process was monitored by detecting the exposure of VAMP to tetanus toxin following disruption of the SNARE complex (open triangles, samples plus Mg^{2+} ATP γ S; open squares, samples plus Mg^{2+} ATP; closed squares, samples plus Mg^{2+} ATP and Ca^{2+}).

- 9 -

° **FIG. 6.** Binding specificity of GST-tagmin to liposomes of different compositions. GST-tagmin was incubated with pure 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine liposomes (PC) or PC containing 1% w/w 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-D-myo-inositol (PI) or 5 1% (w/w) 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-D-myo-inositol-4-phosphate (PIns) or 1% (w/w) 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-D-myo-inositol-4,5-bisphosphate (PIns 4,5-P₂) in the presence or absence of 10 100 μM free calcium. Liposome binding was quantified by liquid scintillation counting of the [³H]PC used as tracer and expressed as % of total radioactivity used. The unspecific binding of liposomes to the beads was determined by using pure GST and the results were subtracted from the total binding.

15 **FIG. 7.** The binding of PIns-P₂ on synaptotagmin is localized to the carboxy-terminal domain. GST-tagmin, its amino terminal (C2A) and carboxy-terminal (CB2) domains were incubated with PC and 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine] (PS) (3:1 w/w) (upper panel) or with PC 20 liposomes containing 1% (w/w) of PIns-P₂ (lower panel). Liposome binding was determined as in Fig. 6.

25 **FIG. 8.** The binding of tagmin and the tagmin C2B domain to PIns-P₂, but not the calcium-dependent binding of C2A to PS, is inhibited by inositol 1,2,3,4,5,6-hexakisphosphate. Liposome binding was determined as in Fig. 6.

30 **FIG. 9.** Synaptotagmin binds to both PIns-P₂ isomers with similar efficiency. Liposomes of PC containing 1% (w/w) of phosphatidylinositol-4,5-bisphosphate (PIns 4,5-P₂) or phosphatidylinositol-3,4-bisphosphate (PIns 3,4-P₂) were 35 incubated with GST tagmin in the presence or absence of 100 μM free calcium as described in Fig. 6.

- 10 -

Fig. 10. Synaptotagmin binds specifically to PI_nsPs-containing liposomes in a calcium-dependent manner. GST-tagmin was incubated with liposomes containing PC or PC together with distinct phosphoinositides (as indicated) in the presence or absence of calcium ions (shaded bars, 2 mM EGTA; filled bars, 100 μM free Ca²⁺). Lipid binding was quantified by liquid scintillation counting of the radioactive PC used as tracer and expressed as % of total radioactivity used.

Fig. 11. Calcium dependency of PI_ns-4,5-P₂ and PI_ns-3,4,5-P₃ binding to tagmin. GST-tagmin beads were simultaneously incubated with two populations of PC liposomes either containing PI_ns-4,5-P₂, or PI_ns-3,4,5-P₃, at variable Ca²⁺ concentrations (open circles, PI_ns-4,5-P₂; closed squares, PI_ns-3,4,5-P₃).

Fig. 12. The PI_nsPs binding site on tagmin is localized to the C2B domain. GST fusion proteins containing tagmin or its amino terminal (C2A; aa. 96-265; 10 μg) or the carboxy-terminal (C2B; aa. 248-421; 10 μg) domains were incubated with liposomes containing PC and either 25% (w/w) 1,2-dioleoy-sn-glycero-3-phospho-L-serine (PS) (panel A) or 1% (w/w) PI_ns-4,5-P₂ (panel B) or 1% (w/w) PI_ns-3,4,5-P₃ (panel C) (shaded bars, 2 mM EGTA; filled bars, 100 μM free Ca²⁺).

Fig. 13. The binding of PI_ns-4,5-P₂ to recombinant and native tagmin is saturable and is competed by InsP₆. A) GST-tagmin (filled circles) or GST alone (filled triangle) were incubated at increasing concentration of radioactive PI_ns-4,5-P₂ in detergent micelles in the presence of 2 mM EGTA.

- 11 -

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to the process of synaptic release. More specifically, this invention relates to methods and compositions useful for modulating docking of synaptic vesicles to membranes based on the formation of specific protein complexes which, according to the invention, mediate functional docking of synaptic vesicles. The mechanism identified for docking may be modelled in vitro in cell free systems according to the invention by detecting the formation of various protein complexes.

The various aspects of docking which may be modelled in vitro include the initial binding of tagmin and β -SNAP to form a bimolecular complex which further binds NSF to form a trimolecular complex. This trimolecular complex then binds α -SNAP and α -SNAP receptor (SNAREs) to accomplish docking (Figure 1). Inhibition of any of these complexes would decrease or inhibit synaptic transmission. Conversely, enhancement of complex formation would be expected to facilitate synaptic transmission.

The compositions of this invention are cell free protein complexes comprised of proteins involved in binding of synaptic vesicles to plasma membrane associated with synaptic transmission. The complexes of this invention comprise tagmin and β -SNAP (Figure 2) and may optionally contain one or more additional components such as, for example NSF, α -SNAP, SNAREs, (Figure 3) polyphosphoinositols such as, for example, inositol 1,2,3,4,5,6 hexakis phosphate (IP₆) and inositol 1,3,4,5,6 pentakisphosphate (IP₅) or polyphosphoinositides, such as, for example, phosphatidylinositol-4,5-bisphosphate (PIIns-4,5-P₂) or phosphatidylinositol-3,4,5 trisphosphate (PIIns-3,4,5-P₃). One such complex of the invention comprises tagmin (also referred to in the art as synaptotagmin or

- 12 -

o p65) and β -SNAP (soluble NSF attachment protein). Yet another complex of this invention comprises tagmin and PIns-P₂, with or without β -SNAP or tagmin and PIns-P₃, with or without β -SNAP. Another such complex of the invention comprises tagmin, β -SNAP and NSF (N-ethylmaleimide-sensitive fusion protein). Still another complex of the invention comprises a quaternary complex of β -SNAP/tagmin/NSF and α -SNAP. Another complex comprises the quaternary complex bound to one or more SNARE proteins. The formation of these complexes in vitro provide the basis for assays useful for identifying substances which modulate synaptic transmission and would therefore be useful as therapeutics or diagnostic reagents.

As used in this invention, "tagmin" refers to any amino acid sequence which comprises at least, the portion of the carboxy terminal region of tagmin which binds β -SNAP. Other amino acid sequences which are sufficiently homologous to this portion of the amino acid sequence of tagmin and which retain the ability to bind β -SNAP are also suitable for use in this invention.

Native tagmin isolated from synaptic vesicles (as, for example, the isolation of tagmin I as described in Südhof et al., Neuron 2:1475-1481 (1989)), or recombinant tagmin obtained from cells which have been transformed with a gene encoding tagmin, or a fragment thereof, are suitable for use in this invention. Recombinant tagmin, or fragments thereof, may be expressed either directly or as a fusion protein attached to additional amino acids. Such methods of expressing protein are known in the art and are also described herein in the examples. cDNA cloning and the construction of vectors for expression of tagmins or specific amino acid sequences of various tagmins fused to glutathione S-transferase (GST) has previously been described in Ullrich et al., Neuron, 13:1281-1291 (1994) and Li et al., Nature, 375:594-599 (1995).

Tagmin exists in different isoforms which are

- 13 -

° distributed throughout the nervous system. Ullrich et al., Neuron 13:1281-1291 (1994). Because certain isoforms are specific for particular regions, it is therefore possible, through this invention, to identify substances which modulate synaptic transmission in specific regions of the nervous system. For example, in the rat and mouse brain, isoforms of tagmin have been described, synaptotagmins I to VIII. Synaptotagmins I to V are localized at the level of the nervous system. Synaptotagmins I and II are the two more abundant isoforms and are generally distributed in the rostral and caudal brain regions respectively. 5 Synaptotagmins III and IV are reported to be coexpressed in neurons expressing synaptotagmins I and/or II, but also exhibit differences in regional distribution. Whereas synaptotagmin IV is reported to be distributed throughout the nervous system, synaptotagmin III exhibits pronounced regional differences. Synaptotagmin III is expressed at appreciable levels in the cortex, hippocampus and olfactory bulb and at higher levels in neurons of the basal ganglia and thalamus. Other regions exhibiting appreciable expression of synaptotagmin III include 10 neurons of the midbrain, caudal brainstem, motor nuclei magnocellular part of the red nucleus and the gigantocellular neurons of the reticular formation. Heterogeneity of synaptotagmin III distribution is also 15 observed in the spinal cord where motoneurons exhibited high levels of expression. A more detailed description of the distribution of synaptotagmins is present in Ullrich et al., Neuron, 13:1281-1291 (1994) which is incorporated herein by reference. Based on this regional distribution of tagmins, the present invention provides a means of 20 selectively modulating synaptic transmission by targeting substances for specific tagmin isoforms.

In addition to using the entire tagmin protein, smaller amino acid sequences of tagmin may be used which retain the ability to bind β -SNAP. Specific amino acids 25

- 14 -

may be substituted provided the ability to bind β -SNAP is retained. Based on results described herein, the preferred region of tagmin for use in this invention is the carboxy terminal (C2B) region as this is the region which binds β -SNAP. For synaptotagmin I, fragments comprising the entire cytoplasmic domain, the C2B domain, amino acids 79-421, or 248-421 retain the ability to bind β -SNAP and are preferred. More preferred is the amino acid region of 248-421. Preferred amino acid sequence for other tagmin isotypes are as follows: tagmin II, amino acid residues about 249 to 422; tagmin III, amino acid residues about 405-588; tagmin IV, amino acid residues about 263-425; tagmin V, amino acid residues about 116 to 279; tagmin VI, amino acid residues about 338-511; tagmin VII, amino acid residues about 242 to 403; and tagmin, VIII amino acid residues 177 to 255. The amino acid sequences recited above are based on rat tagmin isoforms but would substantially correspond to the tagmin isoforms of other species as well. The amino acid sequences of the proteins for use in this invention may be modified by substituting amino acids provided they retain the binding characteristics necessary to assess complex formation. In addition, the length of the amino acid fragments described above may be varied again provided binding to β -SNAP remains intact.

β -SNAP is a brain specific 34Kd NSF binding protein capable of binding to SNAP receptors. The amino acid sequence of bovine β -SNAP is known. Whiteheart et al., Nature, 362:353-355 (1993) which is incorporated herein by reference. β -SNAP may be used in accordance with this invention as the full length protein. A peptide corresponding to amino acids 25 to 45 of β -SNAP inhibits the interaction between β -SNAP and tagmin and for this reason, the amino terminal end of β -SNAP is thought to interact directly with tagmin. This preferred peptide could be used to modulate the β -SNAP/tagmin interaction

- 15 -

sequence for use in this invention. Like tagmin, native β -SNAP or a recombinant form may be used.

NSF is a protein which has been described by Wilson et al., Nature, 339:355-359 (1989). The amino acid sequences of NSF from various different animal species are known. Methods of recombinantly expressing NSF have also been described. Wilson et al., Nature, 339:355-359 (1989) and Wilson and Rothman, Meth. Enzymol., 219:309-318 (1990). For use in this invention, NSF may be expressed recombinantly using any of the expression systems available to those in the art including, for example E. coli. In addition, native NSF purified from natural sources may be used.

For use in this invention, the species from which the various proteins are derived is not significant as there is substantial homology across species and complexes can be formed using proteins from different species. For example in the examples described herein, β -SNAP and α -SNAP amino acid sequences are bovine, NSF is a hamster sequence, and tagmin is a rat sequence.

The formation in vitro and in vivo of any of the bimolecular (β -SNAP/tagmin; PIns-P₂/tagmin and PIns-P₃/tagmin), trimolecular (β -SNAP/tagmin/NSF), quaternary (β -SNAP/tagmin/NSF/ α -SNAP) or pentameric (β -SNAP/tagmin/NSF/ α -SNAP/SNAREs) complexes of this invention and their hydrolysis by Mg²⁺ ATP (Figure 5) provides a means for identifying substances which would be expected to be useful to modify synaptic transmission. Thus, substances which decrease or inhibit complex formation in vitro are useful as drug leads from which therapeutics may be identified. Such therapeutics are useful for treating or diagnosing various neurological or psychiatric disorders for which decreasing or inhibiting synaptic transmission generally or in specific regions of the nervous system is desirable. Examples of neurological disorders for which decreasing or inhibiting synaptic

- 16 -

transmission would be expected to be therapeutic include epilepsy which is characterized by improper electrical discharge in one or more brain regions; and diseases such as Huntington's, in which pathology is related to the action of an excitatory neurotransmitter. Bipolar manic-depressive disorder which is currently treated with lithium is another disease for which decreasing synaptic transmission through inhibition of complex formation would be expected to be therapeutic. Pathologies of the neuromuscular junction or pathologies associated with motor neuron function (such as dystonias) may also be related to the physiology of these complexes.

The assays of this invention for identifying substances which modulate synaptic transmission may be carried out using various protocols known to those in the art for measuring binding of molecules to proteins. For the purposes of this invention, any assay capable of detecting relative amounts of complex formation in the presence and absence of test substances is suitable. In one embodiment, one of the complex components is immobilized on a solid support, e.g., agarose beads or immobilized antibodies which is then combined in an appropriate buffer with the other components of the complex in the presence and absence of test substance. The solid support is then separated from the unbound components and the formation of the complex is assessed by detecting the presence of the various components bound to the solid support. After separation of the complex from the free components, the presence of the components in the complex may be detected either by direct analysis of the complex bound to the solid support or following dissociation of the complex and analysis of the liberated components. Presence of β -SNAP, tagmin, NSF or α -SNAP in any of the complexes may be detected, for example by using specific antibodies. Any method known in the art for detecting the presence of a specific antibody may be used

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- 17 -

in the assays of this invention including the use of radioactive isotopes, chemiluminescence or enzymatic action. In the preferred mode of carrying out the assays of this invention, the formation of each form of complex (β -SNAP/tagmin; β -SNAP/tagmin/NSF; β -SNAP/tagmin/NSF/ α -SNAP; or β -SNAP/tagmin/NSF/ α -SNAP/SNARE) is tested separately with test substances to determine the effect on complex formation and on the capability to bind PIns-P₂ or PIns-P₃. Alternatively, it may also be desirable to combine all of the components together in one reaction to assess the overall effect.

In addition to identifying substances which influence formation of the binary, tertiary, quaternary, or pentameric complexes, the present invention also identifies the formation of other complexes which are involved in docking of vesicles to plasma membrane to cause vesicle release. According to the model provided by this invention, prior to vesicle release, the tertiary tagmin/ β -SNAP/NSF complex interacts with α -SNAP and is then capable of binding SNAREs to cause vesicle docking. The binding of α -SNAP to the tertiary tagmin/ β -SNAP/NSF complex to form the quaternary tagmin/ β -SNAP/NSF/ α -SNAP provides another site useful for identifying potentially therapeutic substances and for modulating synaptic transmission. Binding of the tagmin/ β -SNAP/NSF/ α -SNAP complex to a SNARE complex, i.e. docking, provides yet another site for identifying substances which may be useful to inhibit synaptic transmission.

Support that the model provided herein for docking of synaptic vesicles to plasma membranes is predictive for identifying substances which modulate synaptic transmission is provided by the effects of polyphosphoinositols (InsP₄, InsP₅ and InsP₆) on complex formation. As shown in Figure 4, these compounds, which have previously been reported to inhibit synaptic release²¹ and bind tagmin²⁰ inhibited in vitro the formation of the

- 18 -

o cell free tagmin and β -SNAP complexes. Other phosphoinositols besides InsP₄, InsP₅ and InsP₆ and which are identified according to the methods of the present invention are preferred for use in the methods of inhibiting docking and synaptic transmission.

5

Preparation of Complex Specific Antibodies

The complexes of this invention are also useful as immunogens to raise antibodies (polyclonal or monoclonal) specific for each form of complex (β -SNAP/tagmin; β -SNAP/tagmin/NSF; β -SNAP/tagmin/NSF/ α -SNAP; and β -SNAP/tagmin/NSF/ α -SNAP/SNARE). Preferably such antibodies are conformation-specific in that they recognize the functional form of the various complexes of this invention. These antibodies may then be used as specific substances for decreasing synaptic release or as diagnostics for detecting the presence of intracellular complexes. Normally the blood brain barrier would prevent passage of peripherally administered antibodies. However, in various neurological disorders, particularly those associated with trauma which may precipitate improper synaptic release (i.e., epilepsy), the blood brain barrier may be more permeable allowing passage of such peripherally administered antibodies. Alternatively, large molecules such as antibodies may be administered directly into spinal fluid or intrathecally.

The requirement of several proteins to effect docking significantly increases the likelihood that one or more neurological diseases results from improper expression or function of one of these proteins leading to improper complex formation. The antibodies which are provided by this invention may be used to analyze tissue for the presence of appropriate complexes.

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- 19 -

° Binding of tagmin and β -SNAP/tagmin
Complexes to Membrane Phospholipids

Whereas the polyphosphoinositols described above inhibit complex formation, an inositol-containing phospholipid, phosphatidylinositol-4,5-bisphosphate, the isomer phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate present as minor constituents in cellular membranes are compatible with tagmin/ β -SNAP complexes. As used herein, PIns-P₂ refers to either of the phosphatidyl inositol biphosphates, while PIns-P₁ refers to any of the phosphatidylinositol triphosphates. Other analogs of polyphosphatidylinositol may also be suitable for use with this invention and may vary based on the length of the fatty acid chain, for example C₁₆ to C₂₄ and location and number of double bonds or the degree and position of phosphorylation of the inositol ring, although monophosphates appear inactive.

The present invention also relates to a novel calcium sensor and switch important in regulating the priming step of synaptic neurotransmission. Calcium has been identified as the signal for altering the affinity of various inositol polyphosphates. In the absence of free calcium the naturally occurring phosphatidylinositol bisphosphates, PIns-4,5-P₂ or PIns-3,4-P₂, only moderately increase the interaction of tagmin with the liposomes (Fig. 10A). However, under the same conditions, PIns-3,4,5-P₃ strongly promotes the binding of liposomes to tagmin. Fig. 10. The inclusion of phosphatidylinositol (PIns) or phosphatidylinositol-4-phosphate (PIns-4-P) does not result in binding of liposomes to tagmin, either in the presence (100 μ M) or in the absence of free calcium. Fig. 10A.

Calcium (about 100 μ M) switches the specificity of these interactions. Binding of tagmin to PIns-3,4,5-P₃-containing liposomes is greatly reduced, while tagmin

- 20 -

now binds efficiently to both PI_ns-4,5-P₂ and PI_ns-3,4-P₂-containing liposomes. Fig. 10. Qualitatively similar results were obtained by using native tagmin and recombinant tagmin, thus indicating that the conclusions obtained with the GST-tagmin have a general validity
5 regardless of the source of tagmin.

The dependence of PI_ns-4,5-P₂ and PI_ns-3,4,5-P₃ binding on the concentration of free calcium is demonstrated by the present invention (see Fig. 11). Tagmin loses the capacity to bind PI_ns-3,4,5-P₃ between 10 about 0.1 and 1 μM free calcium. It gains the capacity to bind PI_ns-4,5-P₂ progressively above about 1 μM free calcium with maximum binding reached only at about 100 μM. The calcium dependency of the switch of specificity is not significantly affected by altering the concentration of 15 PI_ns-4,5-P₂ or PI_ns-3,4,5-P₃ in the liposome. The presence of magnesium is preferred to maximize the complete switch. A preferred concentration of magnesium is about 0.5-1.0 mM. This calcium switch is useful in modulating the rate 20 of synaptic neurotransmission and provides a valuable target of substances acting on neurotransmitter release. The identification of this calcium switch provides a therapeutic tool for the treatment of various neurological, psychiatric, memory and learning disorders.

Tagmin contains two C2 domains homologous to the 25 calcium and acidic phospholipid binding domain of protein kinase C.^{29,30} The more amino-terminal C2 domain (C2A) binds PS (25 % w/w) in a calcium-dependent manner with a Kd of 3-6 μM.^{27,28} The more carboxy-terminal C2 domain (C2B) binds InsPP and interacts with both PI_ns-P₂ and PI_ns-P₃. While the interaction of the C2B domain with 30 PI_ns-3,4,5-P₃ is reduced by calcium, as for the entire cytoplasmic domain, the interaction of the C2B domain with PI_ns-4,5-P₂ is strong even in the absence of calcium, and only weakly enhanced by calcium, implying cooperativity 35 between the two C2 domains. While the C2A domain is known

- 21 -

° to bind liposomes containing PS in a calcium dependent manner,^{27,28} the C2B domain binds PS-containing liposomes weakly and in a calcium independent manner,^{20,32} further establishing the specificity of the binding to polyphosphoinositides. Fig. 12A.

5 The interaction of both tagmin and C2B with PIns-4,5-P₂ and PIns-3,4,5-P₃-containing liposomes is competitively inhibited by InsP₆, suggesting that all three compounds bind to the same site in the C2B domain. 10 InsP₆ does not affect the calcium-dependent interaction of PS-containing liposomes with tagmin or its C2A domain. In summary, the C2B domain appears to preferentially interact with polyphosphoinositides; however the C2A domain may have some binding activity. For full calcium-sensitivity, it is preferred that the C2A and C2B domains of tagmin be 15 present.

Micellar PIns-P₂ binds to recombinant and native tagmin and is inhibited by PIns-P₆ with an apparent K_d of about 5μM (Fig. 13). The intracellular concentration of, 20 PIns-P₆ is estimated to be in the low micromolar range, which is unlikely to interfere *in vivo* with the binding of tagmin to the polyphosphoinositides described herein. Presynaptic injection of higher concentrations of InsPP causes a reversible blockade of neurotransmitter release²¹. 25 The present findings indicates that InsPP also acts by competing for the binding of tagmin to PIns-4,5-P₂ and/or PIns-3,4,5-P₃.

Binding of PIns-4,5-P₂ and PIns-3,4,5-P₃ to tagmin is highly specific, as shown by 1) lack of tagmin binding to liposomes containing PIns and PIns-4-P; 2) 30 saturable binding of PIns-4,5-P₂ to tagmin with 1:1 stoichiometry; 3) the competitive inhibition of the above binding reactions by the soluble inositol derivative InsP₆; and 4) the switch in specificity from PIns-3,4,5-P₃ to PIns-4,5-P₂ as a function of free calcium ion.

35 As the free calcium concentration is raised from

- 22 -

resting (basal) levels (\leq 30 nM) to about between 0.1 and 10 μ M, tagmin switches its specificity from PIns-3,4,5-P₃ to PIns-4,5-P₂. Many proteins of diverse function are known to contain C2 domains²⁹ or are known to bind InsPP.³¹ Therefore, they, too, have switchable lipid binding specificity, although the switch may be thrown by mechanisms other than calcium binding.

Polyphosphoinositide binding to tagmin and the calcium-dependent switch in its specificity is directly correlated to the mechanism of exocytosis. A PIns-specific transfer protein, a PIns-4-P 5-kinase (synthesizing PIns-4,5-P₂) and PInsP₂ or PInsP, are required for fusion to be triggered by calcium.^{26,36,37} Exocytosis is triggered when free calcium is above a threshold of about 20 μ M,³⁸ the same range at which tagmin binds PS and has switched from binding PIns-4,5-P₂ to PIns-3,4,5-P₃. Fig. 11. The need for PIns-specific transfer protein and PIns-4-P 5-kinase during priming^{26,37} is explained by the need to maintain a pool of PIns-4,5-P₂ to be available to bind tagmin when it releases PIns-3,4,5-P₃. This switch (and the binding to PS) is important in the rapid triggering of exocytosis that occurs after calcium levels rise. It is noted that the C2B domain of tagmin is known to bind β -SNAP and NSF in a Ca²⁺-independent fashion and assemble with them and α -SNAP and SNAREs into a putative docking and fusion particle for exocytosis.³³

Modulation of PIns-P₂ and tagmin binding is another mode of modifying synaptic transmission according to this invention, as PIns-P₂ is involved in secretion of synaptic vesicles. Binding of PIns-P₂ to tagmin also occurs to the carboxy-terminal portion as with β -SNAP and is inhibited by polyphosphoinositols (see Figure 8). Tagmin/ β -SNAP complex binds to PIns-P₂ with the same efficiency as the pure tagmin. Another aspect of this invention is therefore assays which detect changes in

- 23 -

- ° PIns-P₂ binding to the cell free tagmin complexes of this invention and the method of decreasing synaptic transmission by decreasing or inhibiting PIns-P₂ binding to complexes comprising tagmin, β -SNAP or any of the other proteins which participate in docking to SNAREs.
- 5 Substances identified which decrease or inhibit PIns-P₂ and tagmin interactions may be useful to treat neurological or psychiatric diseases in which it is desirable to reduce synaptic transmission. Lithium, a well known drug for the treatment of bipolar manic-depressive disorders, acts by altering the inositol homeostasis and in particular by reducing the PIns-P₂ levels in the membrane. Babarain J.M., Proc. Natl. Acad. Sci., U.S.A., 91:5738-5739 (1994), Gain et al., Biochem. biophys. Acta., 1177:253-269 (1993). This invention
- 10 provides an assay for identifying other such substances which may be useful as adjuncts or in place of traditional therapies for bipolar depression which rely on lithium.
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The phosphatidylinositol pathway which causes phosphorylation at position 3 is significantly different from that resulting in phosphorylation at positions 4 and 5. Enzymes for the synthesis and degradation of these two different PIns-P₂ species are completely separated and non-overlapping in the cell. Accordingly, by targeting modulation of enzymes specific for one PIns-P₂ isomer over the other, this invention provides another means of specifically modulating synaptic transmission.

One embodiment of this invention relates to a method of treatment to improve synaptic transmission by modulating formation of tagmin-containing complexes, wherein calcium sensitivity of PIns-P₂/tagmin and PIns-P₃/tagmin complex formation is modulated by the addition of external substances.

Examples of "external substances" which may be used in this invention include several preferred categories of substances. A first preferred category of

- 24 -

external substances encompasses those that act by altering intracellular calcium concentrations, such as, for example, calcium channel blockers, including phenylalkylamines, dihydropyridines, benzothiazepines and diphenylpiperazines. Other calcium channel blockers are known in the art, such as those described by Goodman and Gilman's The Pharmacological Basic of Therapeutics 8th Eds. ((Eds. Goodman Gilman et al.) Pergamon Press 1990 p. 774-783). A second preferred category of external substances encompasses those that inhibit or enhance mobilization of calcium from intracellular calcium stores. Such substances include, for example, thapsigargin, inositol 1,4,5 trisphosphate ("IP₃") and 2,5-di(tert-butyl)-1,4-benzohydro quinone. Other such compounds are known in the art. A third preferred category of external substances useful in this method of the invention encompass substances which inhibit or enhance the synthesis of polyphosphoinositides and their soluble analogues including IP₆ and its derivatives, for example, lithium which reduces the level IP₃ and of polyphosphoinositides.

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Assay Systems and Kits

The assay systems and kits of this invention comprise the reagents necessary to conduct the various binding assays described above which may be used to identify substances which reduce or inhibit complex formation. A typical assay kit of this invention would comprise β-SNAP or an amino acid sequence of β-SNAP capable of binding to tagmin, one or more containers each comprising an isoform of tagmin or an amino acid sequence of tagmin capable of binding β-SNAP, and optionally containers comprising separately one or more of NSF, α-SNAP, SNAREs complex, labelled specific antibodies, PI_ns-P₂, PI_ns-P₃ and one or more polyphosphoinositols to serve as controls to compare against test substances.

- 25 -

Components of the kits or assay systems may be provided in appropriate buffers, or may be lyophilized for later reconstitution and use. A preferred buffer is HEPES-KOH (20 mM), 100 mM KCl, dithiothreitol (0.1 mM) at pH 7.6. In particular the region of the amino acid sequence of tagmin extending from about residue 248 to about 421 or the entire cytoplasmic domain (amino acids 78-421) are particularly preferred for use in this invention because, as shown below, this region contains the binding site for β -SNAP and are stable in solution.

As discussed above assays are performed according to the invention by combining the complex components in an appropriate buffer, separating bound from free components and assaying for the presence of particular components in the complex. A preferred buffer for the binding reaction is HEPES-KOH (20mM, pH 7.6), potassium acetate (150 mM), glycerol (1%), octylglucoside (0.6 mM) and dithiothreitol (0.1 mM).

EXAMPLES

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EXAMPLE 1. β -SNAP/Taqmin Complexes

In preliminary experiments, α -SNAP or β -SNAP were added to octylglucoside extracts of brain membranes, but only α -SNAP was recovered bound to the SNAREs. This was surprising, as either SNAP isoforms can bind when added to previously isolated SNAREs (Fig. 1c) and suggested that β -SNAP, but not α -SNAP, must bind with high affinity to an abundant membrane protein present in crude extracts but not in pure SNARE preparations. In fact, glutathione-S-transferase (GST)- β -SNAP fusion protein specifically binds a 65 kD polypeptide from crude extracts identified as synaptotagmin (tagmin) by Western blotting.

Confirming this, beads containing the cytoplasmic domain (Fig. 2a) of tagmin (residues 79 - 421

- 26 -

o of tagmin I) linked to GST bind about 1 mole of β -SNAP/mole of tagmin (Fig. 2b, lane 3) at saturation, while neither α -SNAP (lane 2) nor the other ubiquitous SNAP isoform γ -SNAP⁹ bind. Only the carboxy-terminal portion of tagmin (residues 248-421; Fig 2a) containing the C2B domain binds β -SNAP (Fig. 2b, lane 9); the C2A domain-containing amino terminal portion (residues 96-265) that is known to bind calcium and acidic phospholipids¹⁰ does not bind β -SNAP (lane 8).

Tagmin/ β -SNAP/NSF complex is stable in the presence of $\text{Ca}^{2+}/\text{Mg}^{2+}$ ATP (right panel). GST fusion proteins of different tagmin constructs or GST alone were incubated with α -SNAP (lanes 2, 4, 7) or β -SNAP (lanes 3, 5, 7-13) with (lanes 4-7, 10-13) or without (lanes 1-3, 8, 9) addition of NSF. Three samples, identical to the one in lane 5, were further incubated in the presence of ATP γ S (lane 11), ATP (lane 12) or ATP + Ca^{2+} (lane 13). c) α -SNAP and β -SNAP binding to the SNARE complex. SNARE complexes were isolated from bovine brain detergent extract and incubated with increasing amounts of α -SNAP (closed squares) or β -SNAP (open squares).

METHODS: GST-fusion proteins containing the cytoplasmic domain of synaptotagmin I (aa. 79-421; 4 μg) or either the amino-terminal (GST-p65_{1,3}; aa. 96-265; 3 μg) or the carboxy-terminal (GST-p65_{3,5}; aa. 248-421; 3 μg) parts, were immobilized on GSH-agarose beads. Then 3 μg of His₆- α -SNAP³ or 3 μg of His₆- β -SNAP were diluted in 250 μl of 20 mM HEPES-KOH pH 7.6, 150 mM Acetate-K, 1% glycerol, 5 μM EDTA, 0.8% (w/v) octyl- β -D-glucopyranoside, 0.4 mg/ml ovalbumin, spun at 15,000 $\times g$ for 5 min and the supernatant was incubated with beads for 1 h at 4°C. Subsequently the beads were washed twice with 250 μl of the same buffer without ovalbumin. Some samples were further incubated for 1 h at 4°C with 5 μg of recombinant NSF³ in 250 μl of 20 mM HEPES-KOH pH 7.6, 150 mM Acetate-K, 1 mM MgCl₂, 5 μM EGTA, 0.5 mM ADP, 1% glycerol,

- 27 -

° 0.8% (w/v) octyl- β -D- glucopyranoside, 0.4 mg/ml ovalbumin. The beads were then washed in the same buffer without ovalbumin and samples analyzed in SDS-PAGE. The stability of the complex was tested by incubating the complex-bound beads for 30 min at 37°C in 20 mM HEPES-KOH pH 7.6, 150 mM Acetate-K, 1 mM MgCl₂, 1% glycerol, 0.5% (w/v) Triton X-100 (buffer A) containing 0.5 mM adenosine-5'-[γ -thio]triphosphate (ATP γ S)/1 mM EGTA, 0.5 mM ATP/1 mM EGTA, or 0.5 mM ATP/300 μ M free Ca²⁺.

For the determination of the α / β -SNAP binding to the SNARE complex, increasing amounts of SNAPs were diluted in 20 mM HEPES-KOH pH 7.6, 150 mM Acetate-K, 1% glycerol, 0.5% (w/v) Triton X-100 (buffer B), prespun and added to beads containing the SNARE complex isolated from bovine brain extract (250 μ g of protein/sample), by using a monoclonal anti-syntaxin antibody coupled to Protein G Fast Flow⁷. After 1 h at 4°C the beads were isolated, washed and the samples analyzed by Tris-urea/SDS-PAGE³. The amount of recovered proteins was evaluated by densitometric scanning of the Coomassie Blue R-250 stained bands. Data points (Figure 2) represent the average of three independent experiments.

Since NSF is known to bind to membranes in a SNAP-dependent manner,^{11,12} we tested whether NSF might bind to the β -SNAP-tagmin complex. NSF binds efficiently to the tagmin- β -SNAP complex (lane 5), while α -SNAP neither substitutes for β -SNAP (lane 4) nor interferes with this binding (lane 7).

NSF bound through α -SNAP to SNAREs hydrolyses ATP, releasing NSF and α -SNAP, with disruption of the SNARE complex⁷. However, complexes of NSF and β -SNAP with tagmin are stable in the presence of Mg²⁺-ATP, with (lane 13) or without (lane 12) calcium (0.3 mM), and these complexes form in the absence or presence (0.3 mM) of calcium (not shown). The lack of release of NSF with Mg²⁺-ATP suggests that β -SNAP and NSF are bound to tagmin

- 28 -

differently than in 20S particles,^{3,7} and implies that the tagmin/ β -SNAP/NSF "triple" complex should be stable *in vivo* under conditions in which ample ATP is present. Tagaya and colleagues¹³ have reported that NSF is present on synaptic vesicles, and is not released by Mg²⁺-ATP; the tagmin/ β -SNAP/NSF triple complex may explain their findings.

EXAMPLE 2. Evidence For Triple Complex
Involvement In Docking

METHODS: GST, GST-tagmin, GST-tagmin/ β -SNAP and GST-tagmin/ β -SNAP/NSF complexes, bound to GSH-agarose beads were prepared as described in Fig. 2. The complexes were resuspended in 20 mM HEPES-KOH pH 7.6, 150 mM Acetate-K, 1% glycerol, 0.8% (w/v) octyl- β -D-glucopyranoside, (Buffer C) containing 1 mM MgCl₂, 0.5 mM ADP, 1 mM phenylmethylsulfonylfluoride, 1 mM dithiothreitol and mixed in a final volume of 500 μ l with bovine brain extract (250 μ g of protein) prepared as described³ by using octyl- β -D-glucopyranoside as detergent. 10 μ g of His_n- α -SNAP or 10 μ g of His_n- β -SNAP were added as indicated. The samples were incubated for 1 h at 4°C, recovered by centrifugation at 5,000 \times g for 1 min and washed three times in buffer C containing 1 mM MgCl₂, 0.5 mM ADP. The pellets were analyzed by Tris-urea/SDS-PAGE, transferred to nitrocellulose and immunodecorated with a monoclonal antibody against syntaxin/HPC1 and with polyclonal antibodies against SNAP-25 and against rat VAMP⁷. Immunoreactive bands were visualized by using the Enhanced Chemoluminescence method (ECL, Amersham). Similar results were obtained by using Triton X-100 bovine membrane extract³ and by controlling the free calcium concentration to 1 μ M as described for calcium-dependent priming²³.

Consistent with this, assembled SNARE complexes

- 29 -

° bind to the triple complex immobilized on beads, but only when α -SNAP was also added (Fig. 3, lane 8), underscoring the specificity of this interaction. Much less binding occurred in the absence of α -SNAP (lane 7). β -SNAP could substitute for α -SNAP but was not as potent (lane 9). In 5 keeping with this, β -SNAP binds to SNAREs (Fig. 2c, open squares) with 2-3-fold lower affinity than does α -SNAP (closed squares); similar amounts of each (about 3 moles per mole of SNAP-25) bind at saturation. All three SNARE proteins (VAMP, syntaxin, and SNAP-25) bind together (lane 10 8), along with α -SNAP (not shown); the previously associated β -SNAP and NSF remain bound (not shown). Efficient binding of α -SNAP-SNARE complexes only occurred when NSF was present in the triple complex, and not to beads containing just GST-tagmin, or GST-tagmin- β -SNAP 15 complexes (Fig. 3), and was not significantly affected by calcium.

Previously, it was reported⁷ that endogenous tagmin, isolated in low molar yield bound to the native synaptic SNARE complex, was displaced by excess α -SNAP, leading to the hypothesis that the SNARE complex could not simultaneously bind both tagmin and NSF/SNAPS. The recombinant tagmin cytoplasmic domain also binds weakly to the SNARE complex (Fig. 3, lane 1) and is also displaced by excess α -SNAP. Thus, the present data does not 20 contradict but rather extend the previous findings with the important addition that it is the highly specific interaction of tagmin with β -SNAP that allows the SNARE complex to simultaneously bind tagmin, β -SNAP and NSF. 25

Because each of its subunits or close homologue 30 is known to be required *in vivo* for exocytosis according to physiological^{4,18}, pathophysiological¹⁹ or genetic tests^{6,8,16}, this complex represents a specialized docking and fusion particle for regulated exocytosis at the synapse, and perhaps elsewhere.

- 30 -

EXAMPLE 3. Inhibition Of β -SNAP/Tagmin Complex Formation by Polyphospho-inositols.

Since it was found that the C2B domain also binds β -SNAP (Fig. 2b, lane 9), the effects of polyphospho-inositol compounds on the binding of β -SNAP to tagmin were tested (Fig. 4).

METHODS: 4 μ g of GST-tagmin immobilized on GSH-agarose beads were incubated for 15 min at 4°C with variable concentration of inositol 1,4,5-trisphosphate (InsP_3) or inositol 1,3,4,5-tetrakisphosphate (InsP_4) or inositol 1,3,4,5,6-pentakisphosphate (InsP_5) or inositol 1,2,3,4,5,6-hexakisphosphate (InsP_6) (Sigma). Then β -SNAP (4 μ g) diluted in buffer C containing 0.4 mg/ml ovalbumin and previously spun at 15,000 \times g for 5 min was added. The samples (250 μ l final volume) were incubated for 1 h at 4°C, washed twice with 250 μ l of buffer C and then analyzed by SDS-PAGE. The β -SNAP bound to GST-tagmin was determined by scanning of the Coomassie Blue R250 stained gel as described before (see legend Fig. 2).

These same compounds blocked β -SNAP binding with the same rank order and with similar potency to that reported for their binding to tagmin²⁰. Preformed tagmin- β -SNAP and tagmin- β -SNAP-NSF triple complexes are stable in the presence of these substances. This implies that active polyphosphoinositols introduced *in vivo* would only block the assembly of new triple complexes and new docking and fusion particles formed from them. Consistent with this, polyphosphoinositols potently blocks transmission when injected presynaptically, following a lag time of 15-45 min²¹. While this can be explained by the effects of these compounds on the binding of β -SNAP to tagmin, additional effects in synapses cannot be excluded.

- 31 -

° EXAMPLE 4. Mg²⁺ ATP Dependent Disassembly Of the Specialized Docking And Fusion Particle

NSF entering the possible docking and fusion particle bound in the "triple" complex with β -SNAP and tagmin can hydrolyze ATP to release VAMP as shown by cleavage by tetanus toxin²² (Fig. 5, open squares). ATP γ S does not substitute for ATP (open triangles), and an ATP-hydrolysis deficient mutant of NSF (E329Q/D604Q double mutant)¹¹ will substitute for NSF in assembling the particle, but produce particles resistant to Mg²⁺ATP. These results imply that this new docking and fusion particle is not a dead-end complex, i.e., a particle containing a non-functional NSF molecule unable to disrupt the SNARE complex.

METHODS: The complex of GST-tagmin/ β -SNAP/NSF with SNAREs and α -SNAP was assembled from Triton X-100 bovine brain membrane extract³ as described in Fig. 1 and 2 and washed twice with buffer B, containing 1 mM MgCl₂ and 0.5 mM ADP. The particle-containing beads (15 μ l per sample) were then added to 235 μ l of pre-warmed buffer A supplemented with 400 nM of the recombinant catalytic subunit of tetanus toxin²² and containing alternatively 0.5 mM ATP γ S/1 mM EGTA, 0.5 mM ATP/1 mM EGTA, or 0.5 mM ATP/300 μ M free Ca²⁺ (obtained by buffering with 1 mM EGTA). At the indicated times samples were chilled on ice and centrifuged at 5,000 \times g for 1 min. Pellets were analyzed by Tris-urea/SDS-PAGE, transferred to nitrocellulose and immunodecorated with a polyclonal antibody against rat VAMP⁷. Immunoreactive bands were visualized by ECL and quantified by scanning of the resulting X-ray film. VAMP content at different incubation times was expressed as percentage of the amount present at time zero. Analysis of supernatants showed only trace amounts of VAMP independent of the time of incubation, as expected.

Calcium does not affect the rate of ATP-

- 32 -

o dependent disassembly of the particle (Fig. 5, closed squares). Studies of calcium-triggered exocytosis in patch-clamped and in permeabilized neuroendocrine cells have revealed two distinct stages in this process^{23,25}. A slow ATP-dependent, but calcium-independent process termed 5 "vesicle priming", is followed by a rapid ATP-independent step triggered by calcium.

Our observations favor the possibility that priming includes the NSF-ATPase-dependent disruption of the proposed synaptic docking and fusion particle, 10 producing a latent fusogenically-active state awaiting calcium. If this assignment is correct, then, like priming, disruption of the docking and fusion particle should be independent of calcium, as we observed (Fig. 5). This view in no way contradicts the additional need for 15 ATP during the priming stage to maintain a pool of phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P₂) for later fusion after calcium activation²⁶. The protein components that might constitute the latest fusogenically-active state that be produced when ATP is hydrolyzed by 20 NSF are unknown but could be satisfied by tagmin which possesses a calcium binding site and an affinity for β -SNAP.

EXAMPLE 5. Binding Of Liposomes to Complexes

25 Methods: Liposomes (175 μ g/ml) were made by pure 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (PC) or PC and 1,2-dioleoy-sn-glycero-3-[phospho-L-serine] (PS) (3:1 w/w) or PC containing 1% w/w 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-D-myo-inositol (PI) or 1% (w/w) 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-D-myo-inositol-4-phosphate (PIns-P) or 1% (w/w) 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-D-myo-inositol-4,5-bisphosphate (PIns 4,5-P₂). Liposomes contained 1 μ Cl/ml 30 of 1,2-dipalmitoyl-sn-glycero-3-phospho-[N-methyl-3H]-

- 33 -

o choline (66 Ci/mmol, Amersham) as tracer. Lipids were purchased from Avanti Polar Lipids (PC, PS and PI) or Boehringer Mannheim GmbH (PIns-P and PIns-P₂). Phospholipids were dried by a gentle flow of argon on a 4 ml glass vial and then the residual traces of solvents were eliminated under vacuum for 15 minutes at room temperature. The lipid film were resuspended in 20 mM HEPES-KOH pH 7.6, 100 mM KC1 mM DTT (buffer A) by vigorous stirring for 10 minutes and the top of the solution were layered with argon. Liposomes were prepared either by sonication in a Branson cup sonicator (intensity setting 7, duty cycle 50%, 8 minutes)²⁸ followed by liposomes purification on Sephadex G-25 M (Pharmacia) or by freeze-thawing (5 times) and extrusion (19 times) through a 100 nm pore carbonate membrane (Avestin, Ottawa, Canada)⁴². Large aggregates were eliminated by centrifugation at 15,000g for 10 minutes. The liposomes are stored at 4°C and are used within one week. The FPLC-purified cytoplasmic domain of synaptotagmin 1 (tagmin, aa. 79-421; 15 µg) or its fragments, correspondent to the amino-terminal (C2A; aa. 96-265; 11 µg) and the carboxy-terminal (C2B; aa. 248-421; 11 µg) portions, fused with glutathione-S-transferase (GST) or GST alone (8 µg) were immobilized on 20 µl 50% GSH-agarose beads and washed twice with 500 µl of buffer A containing alternatively 2 mM EGTA or 100 µM free calcium buffered with 2 mM EGTA. Subsequently 100 µl of [³H]-labelled liposomes containing the desired concentration of free calcium were added and the beads were incubated for 20 minutes at room temperature with vigorous shaking. In selected samples, liposome binding were performed in the presence of 5 µM inositol 1,2,3,4,5,6-hexakisphosphate (InSP6, Sigma). Samples were centrifuged at 3000g for 2 minutes and the pellets were washed three times with buffer A plus or minus 100 µM free calcium. The bound liposomes were solubilized with 0.4 ml of 10% SDS and radioactivity

- 34 -

° quantified by scintillation counting. The experimental findings were confirmed in at least three independent experiments. The molecular identity of the inositol phospholipids were confirmed by mass spectrometry and by thin-layer chromatography-immunostaining assay (Higashi et al., J. Biochem. (Tokyo) 95, 1517 1984) with an anti-PI_{n-P₂} specific antibody (Matuoka et al., Science 239:640-643 (1988)).

As shown in Fig. 6, only liposomes containing PI_{n-P₂} bound significantly to tagmin, other inositol containing phospholipids (PI and PI_{n-P}) show only minor binding activity. Evidence that PI_{n-P₂} binds to the same region of tagmin as does β -SNAP is provided by the inhibition by inositol 1,2,3,4,5,6-hexakisphosphate (IP₆) of PI_{n-P₂} binding to tagmin (Fig. 8B). The lack of any inhibition of PS binding by IP₆ further confirms the different specificity of binding. The PI_{n-P₂} binding site is localized to the carboxy-terminal domain (C2B) and accounts for the entire PI_{n-P₂} binding of synaptotagmin. In fact, the amino terminal domain, C2A, shows barely detectable binding activity which is not distinguishable from the fusion protein alone (GST) (Fig. 7, lower panel). The functionality of the C2A domain is demonstrated from the calcium-dependent binding of this region of synaptotagmin (Fig. 7, upper panel).

25
EXAMPLE 6. Specific Calcium-dependent Binding of Synaptotagmin to PI_{nPs}-Containing Liposomes.

To test if the InsPP binding site on tagmin can also bind corresponding lipid phosphoinositides, beads containing glutathione S-transferase (GST) linked to tagmin I (residues 79-421) were incubated with liposomes containing phosphatidylcholine (PC) and different inositol phospholipids (1 % (w/w)) at close to the physiological concentration of free Mg²⁺ (0.5-1 mM).

- 35 -

METHODS: Liposomes (175 µg of lipid/ml) were made from pure 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (PC) or PC and in addition 1% (w/w) of 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-D-myo-inositol (PIns) or 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-D-myo-inositol-4-phosphate (PIns-4-P) or 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-D-myo-inositol-4,5-bisphosphate (PIns-4,5-P₂) or 1,2 dipalmitoyl sn-glycero-3-phospho-D-myo-inositol -3,4-bisphosphate (PIns-3,4-P₂ or 1,2 dipalmitoyl sn-glycero-3-phospho-D-myoinositol -3,4,5-trisphosphate (PIns-3,4,5-P₃) and 1 µCi/ml of 1,2-dipalmitoyl-sn-glycero-3-phospho-[N-methyl-³H]-choline (66 Ci/mmol, Amersham) was added as a tracer. Lipids were purchased from Avanti Polar Lipids (PC and PIns) or Boehringer Mannheim GmbH (PIns-4-P and PIns-4,5-P₂) or Matreya Inc (PIns-3,4-P₂). PIns-3,4,5-P₃ was synthesized as described before⁴¹ and its identity was confirmed by ¹H- and ³¹P-NMR. Lipids were dried by a gentle flow of argon, dissolved in 200 µl of 100 % ethanol and then kept under vacuum for 30 min. at room temperature. The homogeneous lipid film was then resuspended in 20 mM HEPES-KOH pH 7.6, 100 mM KCl, 0.2 mM dithiothreitol (DTT) (buffer A) by vigorous stirring for 10 minutes, after overlaying the top of the solution with argon. Liposomes were prepared either by sonication²⁸ followed by liposome purification on Sephadex G-25 M (Pharmacia) or by extrusion⁴². Large aggregates were eliminated by centrifugation at 15,000g for 10 minutes. Purified GST-fusion proteins containing the cytoplasmic domain of tagmin 1 (aa. 79-421; 15 µg) or GST alone (8 µg) were immobilized on 20 µl 50% glutathione-agarose beads (Sigma). The beads were then washed twice with 500 µl of the same buffer containing 1.0 mM free Mg²⁺ and either 2 mM EGTA or 100 µM free calcium buffered with 2 mM EGTA. Subsequently, 100 µl of [³H]-labeled liposomes were added and the beads were incubated for 30 minutes at room

- 36 -

° temperature with vigorous shaking. Beads were centrifuged at 3000g for 2 minutes and washed three times with buffer A containing 1.0 mM free Mg²⁺ plus or minus 100 μM free calcium. The bound liposomes were solubilized with 0.3 ml of 10% SDS and radioactivity quantified by scintillation counting. Data shown are the average of three independent experiments ± s.d.

5 Figure 10A shows the binding specificity of GST-tagmin to liposomes. GST-tagmin was incubated with liposomes containing PC or PC together with distinct phosphoinositides (as indicated) in the presence or absence 10 of calcium ions (shaded bars, 2 mM EGTA; filled bars, 100 μM free Ca²⁺). Lipid binding was quantified by liquid scintillation counting of the radioactive PC used as tracer and expressed as % of total radioactivity used. 15 Specific binding was calculated by subtracting the non-specific lipid interaction of GST (2,4± 0.7%) from individual samples. Similar results were obtained both with small and large unilamellar vesicles, thus suggesting 20 that size and curvature of the liposome do not influence the binding.

EXAMPLE 7. Calcium dependency of PIIns-4,5-P₂ and PIIns-3,4,5-P₃ binding to tagmin.

25 PIIns-4,5-P₂ and PIIns-3,4,5-P₃ were incorporated into separate liposome populations, labeled either with a [¹⁴C] or with a [³H]-PC tracer. Binding of each vesicle type to tagmin-containing beads was determined in a mixed 30 incubation as a function of free calcium concentration (Fig. 11). GST-tagmin beads were simultaneously incubated with two populations of PC liposomes either containing PIIns-4,5-P₂ or PIIns-3,4,5-P₃ at variable Ca²⁺ concentrations (See Figure 11, open circles, PIIns-4,5-P₂; closed squares, PIIns-3,4,5-P₃).

35 METHODS: PC liposomes (350 μg of lipid/ml) containing either 1% (w/w) of PIIns-4,5-P₂ and 0.1 μCi/ml

- 37 -

° of 1-palmitoyl-2-[1-¹⁴C]palmitoyl-sn-glycero-3-phosphocholine (55 mCi/mmol, Amersham) or 1% (w/w) of PIns-3,4,5-P₃ and 1 μ Ci/ml of 1,2-dipalmitoyl-sn-glycero-3-phospho-[N-methyl-³H]-choline (66 Ci/mmol, Amersham) were prepared as above. The two liposome populations were pre-mixed at a 5 1:1 ratio (final lipid concentration 175 μ g/ml) and free Ca²⁺ concentrations ranging from 1 nM to 300 μ M in the presence of 0.5 mM free Mg²⁺ were obtained by adding the suitable amount of calcium or magnesium chloride to 2 mM EGTA in buffer A. GST-tagmin beads were then incubated 10 with the mixed liposomes for 30 min. at room temperature and washed in buffer A containing the corresponding amount of free Ca²⁺. [³H]/[¹⁴C] radioactivity associated with the pellet was then determined by scintillation counting.

Tagmin loses the capacity to bind PIns-3,4,5-P₃ between about 0.1 and 1 μ M free calcium. It gains the capacity to bind PIns-4,5-P₂ progressively above about 1 μ M free calcium with maximum binding reached only in the 100 μ M range. The calcium dependency of the switch of specificity was not significantly affected by altering the 20 concentration of PIns-4,5-P₂ or PIns-3,4,5-P₃ in the liposome. However, magnesium (0.5-1.0 mM) was required for the complete switch. In the absence of Mg²⁺, the calcium dependency of the binding of tagmin to PIns-4,5-P₂-containing liposomes was less pronounced, as was the 25 calcium dependent reduction of the interaction of PIns-3,4,5-P₃ with tagmin.

EXAMPLE 8. Tagmin domains and Polyphosphoinositides binding specificity.

30 Beads containing bacterially expressed GST-tagmin, its GST-C2A or its GST-C2B domains were incubated with liposomes containing 1 % (w/w) PIns-4,5-P₂ or PIns-3,4,5-P₃ (Fig. 12).

35 The PInsPs binding site on tagmin is localized

- 38 -

o to the C2B domain. GST fusion proteins containing tagmin or its amino terminal (C2A; aa. 96-265; 10 μ g) or the carboxy-terminal (C2B; aa. 248-421; 10 μ g) domains were incubated with liposomes containing PC and either 25% (w/w) 1,2-dioleoy-sn-glycero-3-phospho-L-serine (PS) (Figure 12, panel A) or 1% (w/w) PIns-4,5-P₂ (Figure 12, panel B) or 1% (w/w) PIns-3,4,5-P₃ (Figure 12, panel C) (shaded bars, 2 mM EGTA; filled bars, 100 μ M free Ca²⁺). Liposome binding to tagmin and its domains was determined as in Fig. 10A.

10 The PIns-4,5-P₂ and the PIns-3,4,5-P₃-directed binding of liposomes occur with the C2B domain of tagmin, with an efficiency similar or higher to the full length tagmin cytoplasmic domain (Fig. 12B and C).

15 EXAMPLE 9. Binding Kinetics of Micellar PIns-4,5-P₂ to Recombinant and Native Tagmin.

20 The binding of individual molecules of PIns-4,5-P₂ (as distinct from liposomes containing PIns-4,5-P₂) to tagmin could be measured directly because of the availability of radiolabeled PIns-4,5-P₂.

25 GST-tagmin (Fig. 13, filled circles) or GST alone (Fig. 13, filled triangles) were incubated at increasing concentration of radioactive PIns-4,5-P₂ in detergent micelles in the presence of 2 mM EGTA, as shown in Figure 13A. Samples were analyzed as described in Example 6. Panel A of Fig. 13 shows the tagmin/PIns-4,5-P₂ molar ratio as function of the micellar PIns-4,5-P₂ concentration. Dashed line (open circles) represents the PIns-4,5-P₂ specifically associated with tagmin. Parallel experiments using Triton X-100 (0.02% w/v) gave the same results.

30 35 GST-tagmin was incubated in the presence of saturable amount of PIns-4,5-P₂ (6 μ M) with increasing amount of InSP₆ in the presence of 2 mM EGTA, as shown in

- 39 -

° **Figure 13B.**

Immunoprecipitated native tagmin (Fig.13C, filled circles) or anti-tagmin antibody alone (Fig.13C, filled triangles) were incubated with radioactive PIns-4,5-P₂ in detergent micelles in the presence of 2 mM EGTA, 5 as shown in panel C. Dashed line (open circles) represents the PIns-4,5-P₂ specifically associated with tagmin.

SDS-PAGE profile of the immunopurified native tagmin used in panel C of Figure 13. In addition to the tagmin monomer with an apparent molecular weight (Mr) of 65 kDa, a SDS-resistant tagmin dimer is also visible⁴⁰.

METHODS: For the determination of the apparent affinity constant and the total number of binding sites for PIns-4,5-P₂ on tagmin, 1,2-dioleoy-sn-glycero-3-phospho-D-myo-inositol-4,5-bisphosphate [myo-inositol-2-³H(N)] (7 Ci/mMole; NEN) and unlabelled PIns-4,5-P₂ were mixed and dried with a gentle flow of argon. The lipid film was then dissolved in 200 µl of 100 % ethanol and the trace amounts of solvent were eliminated under vacuum. PIns-4,5-P₂ was solubilized at a final concentration of 20 µM (20 Ci/Mole) in buffer A containing 0.8 % (w/v) OG in the presence of 2 mM EGTA and sonicated as above. For the experiments shown in Fig. 13, panels C) and D) native tagmin (5 µg/sample) was immunoprecipitated from octyl-β-D-glucopyranoside (OG) extract of bovine brain cortex³⁵ with an anti-tagmin monoclonal antibody (M48)³³ covalently coupled to protein G-Sepharose Fast Flow (Pharmacia)³. After overnight incubation at 4°C, the beads were washed extensively with buffer A containing 0.5 M NaCl, 0.8 % OG and then rinsed in buffer A containing 0.8 % OG. Monomeric PIns-4,5-P₂ in detergent micelles were mixed with immunopurified native tagmin or immobilized GST-tagmin (6 µg) or control beads, incubated 30 min. at 4°C in the presence of 2 mM EGTA and then analyzed as

- 40 -

described in Example 6.

For Fig. 13D, beads were analyzed by SDS-PAGE and the proteins were stained with Coomassie Blue R. Competition experiments were carried out by incubating GST-tagmin-containing beads with saturable amount of [³H]-PIns-4,5-P₂ (6 μ M) premixed with increasing concentrations of InsP₆ (1 nM-1 mM) in buffer A containing 0.8 % OG, 2 mM EGTA for 30 minutes at 4°C. After washing with the same buffer, the amount of radioactivity was determined and results expressed as % of the binding in absence of InsP₆.

Radiolabeled PIns-4,5-P₂ was dispersed in octyl- β -D-glucopyranoside (OG) micelles using a vast excess of detergent to ensure that there would be at most one molecule of PIns-4,5-P₂ per detergent micelle. At saturation, almost exactly one mole of PIns-4,5-P₂ was bound per mole of tagmin (Fig. 13A), with an apparent Kd of about 1 μ M. Unlike the binding of tagmin to PIns-4,5-P₂ contained in lipid bilayer vesicles, the binding of micellar PIns-4,5-P₂ monomers was independent of calcium. InsP₆ competes with this binding, with a Ki of about 10 μ M. Fig. 13B. Full length native tagmin, immunopurified from brain cortex with an antibody directed against its first C2 homology domain¹³ (Fig. 13D) shows the saturable and stoichiometric binding properties for PIns-4,5-P₂ (Fig. 13C) as was observed with the recombinant protein. InsP₆ inhibits the interaction of micellar PIns-4,5-P₂ monomers with the native protein, but less efficiently than observed with the recombinant tagmin (Ki \geq 100 μ M).

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- 41 -

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10 All of the foregoing references cited herein are incorporated herein by reference in their entirety.

While we have hereinbefore described a number of embodiments of this invention, it is apparent that the basic constructions can be altered to provide other 15 embodiments which utilize the methods and devices of this invention. Therefore, it will be appreciated that the scope of this invention is defined by the claims appended hereto rather than by the specific embodiments which have been presented hereinbefore by way of example.

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- 44 -

WE CLAIM:

1. A cell free protein complex comprising an amino acid sequence of tagmin bound to at least one other molecule selected from the group consisting of PIns-P₂, 5 PIns P₃ and an amino acid sequence of β -SNAP which binds tagmin.

2. The complex according to claim 1 comprising a tagmin binding amino acid sequence of β -SNAP and a β -SNAP binding sequence of tagmin. 10

3. The complex according to claim 2 further comprising at least one other protein selected from the group consisting of NSF, α -SNAP, syntaxin, VAMP, SNAP25, 15 PIns-P₂ and PIns-P₃.

4. The complex according to claim 2 wherein the amino acid sequence of beta-SNAP is bound to the carboxy terminal portion of the tagmin amino acid sequence. 20

5. The complex according to claim 4 wherein the carboxy terminal portion of tagmin bound to beta-SNAP comprises an amino acid sequence selected from the group 25 consisting of amino acid residues about 79-421 of tagmin I, amino acid residues about 248-421 of tagmin I, amino acid residues about 249 to 422 of tagmin II; amino acid residues about 405-588 of tagmin III; amino acid residues about 263-425 of tagmin IV; amino acid residues about 116 to 279 of tagmin V; amino acid residues about 338-511 of 30 tagmin VI; amino acid residues about 242 to 403 of tagmin VII; and amino acid residues 177 to 255 of tagmin VIII.

- 45 -

6. The complex according to claim 5 wherein the carboxy terminal portion of tagmin I bound to beta-SNAP comprises amino acid residues about 248 to 421.

5 7. The complex according to claim 1 wherein the amino acid sequence of the tagmin comprises the full length sequence of the tagmin.

10 8. The complex according to claim 1 wherein the amino acid sequence of β -SNAP comprises the full length sequence of β -SNAP.

15 9. The complex according to claim 8 wherein the amino acid sequence of β -SNAP comprises amino acid 25 to 45 of β -SNAP.

10. The complex according to claim 5 wherein the tagmin amino acid sequence is provided as a recombinantly expressed fusion protein.

20 11. The complex according to claim 5 further comprising NSF.

25 12. The complex according to claim 1 wherein the tagmin amino acid sequence is bound to a solid support.

13. The complex according to claim 3 comprising β -SNAP, tagmin and NSF.

30 14. The complex according to claim 3 comprising β -SNAP, tagmin, NSF and α -SNAP.

35 16. The complex according to claim 1 comprising β -SNAP, tagmin, NSF, α -SNAP and at least one SNARE protein.

- 46 -

17. The complex according to claim 1 comprising tagmin and PIns-P₂ or tagmin and PIns-P₃.

18. A kit for identifying compounds capable of inhibiting synaptic transmission, said kit comprising a
5 first container comprising an amino acid sequence of beta-SNAP capable of binding to a tagmin and at least one container comprising a beta-SNAP binding protein comprising an amino acid sequence present in at least one isoform of tagmin which amino acid sequence binds beta-
10 SNAP, and wherein the beta-SNAP and beta-SNAP binding protein are present in amounts sufficient to assess the formation of beta-SNAP and tagmin complexes in the presence of a test compound.

15 19. The kit according to claim 18 comprising a plurality of containers comprising beta-SNAP binding protein, each container comprising an amino acid sequence of a different isoform of tagmin.

20 20. The kit according to claim 18 further comprising additional proteins for assessment of complexing formation, said proteins being selected from the group consisting of NSF, α -SNAP and SNARE proteins in amounts sufficient to form complexes with the beta-SNAP
25 and the amino acid sequence of tagmin.

21. The kit according to any one of claims 18,
18 or 19 wherein the beta-SNAP binding amino acid sequence is bound to a solid support.

30 22. The kit according to claim 18 wherein the amino acid sequence of tagmin isoforms is a fragment of tagmin consisting essentially of the carboxy terminal beta-SNAP binding segment.

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- 47 -

° 23. The kit according to claim 22 wherein the amino acid sequence of tagmin consists essentially of amino acid residues about 248 to about 421 of tagmin I.

5 24. The kit according to claim 18 wherein the amino acid sequence of tagmin is present as a fusion protein.

10 25. A method of identifying substances capable of inhibiting synaptic transmission, said method comprising:

15 (a) combining the components to form any of the complexes selected from the group consisting of β -SNAP/tagmin; β -SNAP/tagmin/NSF; β -SNAP/tagmin/NSF/ α -SNAP; β -SNAP/tagmin/NSF/ α -SNAP/SNARE protein; PIns-P₂/tagmin; and PIns-P₃/tagmin with a test substance under conditions which allow for complex formation; and

(b) detecting the formation of complexes.

20 26. The method according to claim 25 wherein a plurality of different isoforms of tagmin are tested for complex formation with beta-SNAP in the presence of the test substance.

25 27. The method according to claim 25 wherein the beta-SNAP binding protein is bound to a solid support.

30 28. The method according to claim 26 wherein the beta-SNAP binding protein is a fusion protein consisting essentially of the carboxy terminal portion of tagmin and an additional amino acid sequence.

35 29. The method according to claim 28 wherein the carboxy terminal portion of tagmin consists essentially of amino acid residues about 248 to about 421 of tagmin I.

- 48 -

30. The method according to claim 29 wherein additional amino acid sequence bound to the carboxy-terminal sequence of tagmin is a GST sequence.

5 31. A method of modulating synaptic transmission comprising exposing a neuronal cell to a substance which inhibits binding of beta-SNAP to tagmin.

10 32. A method of modulating synaptic transmission comprising exposing a neuronal cell to a substance which inhibits binding of PIns-P₂ to tagmin.

33. The method of claim 32 wherein the substance inhibits binding of PIns-P₂ to tagmin.

15 34. A method of modulating synaptic transmission comprising exposing a neuronal cell to a substance which inhibits formation of a complex selected from the group consisting of β-SNAP/tagmin; β-SNAP/tagmin/NSF; β-SNAP/tagmin/NSF/α-SNAP; and β-SNAP/tagmin/NSF/α-SNAP/SNARE protein; PIns-P₂/tagmin; and PIns-P₃/tagmin.

20 25 35. A method of treating a neurological or psychiatric disorder related to improper synaptic transmission by modulating formation of tagmin complexes.

30 36. The method according to claim 35 wherein the tagmin complexes are selected from the group consisting of β-SNAP/tagmin; β-SNAP/tagmin/NSF; β-SNAP/tagmin/NSF/α-SNAP; and β-SNAP/tagmin/NSF/α-SNAP/SNARE protein; PIns-P₂/tagmin; and PIns-P₃/tagmin.

35 37. The method according to claim 35 wherein modulation of tagmin complex formation is affected by altering calcium levels by means of altering permeability

- 49 -

° of a calcium channel.

38. The method according to claim 35 wherein
calcium sensitivity of PI_ns-P₂/tagmin and PI_ns-P₃/tagmin
complex formation is modulated by addition of external
5 substances.

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Assembly of a Synaptic Exosome

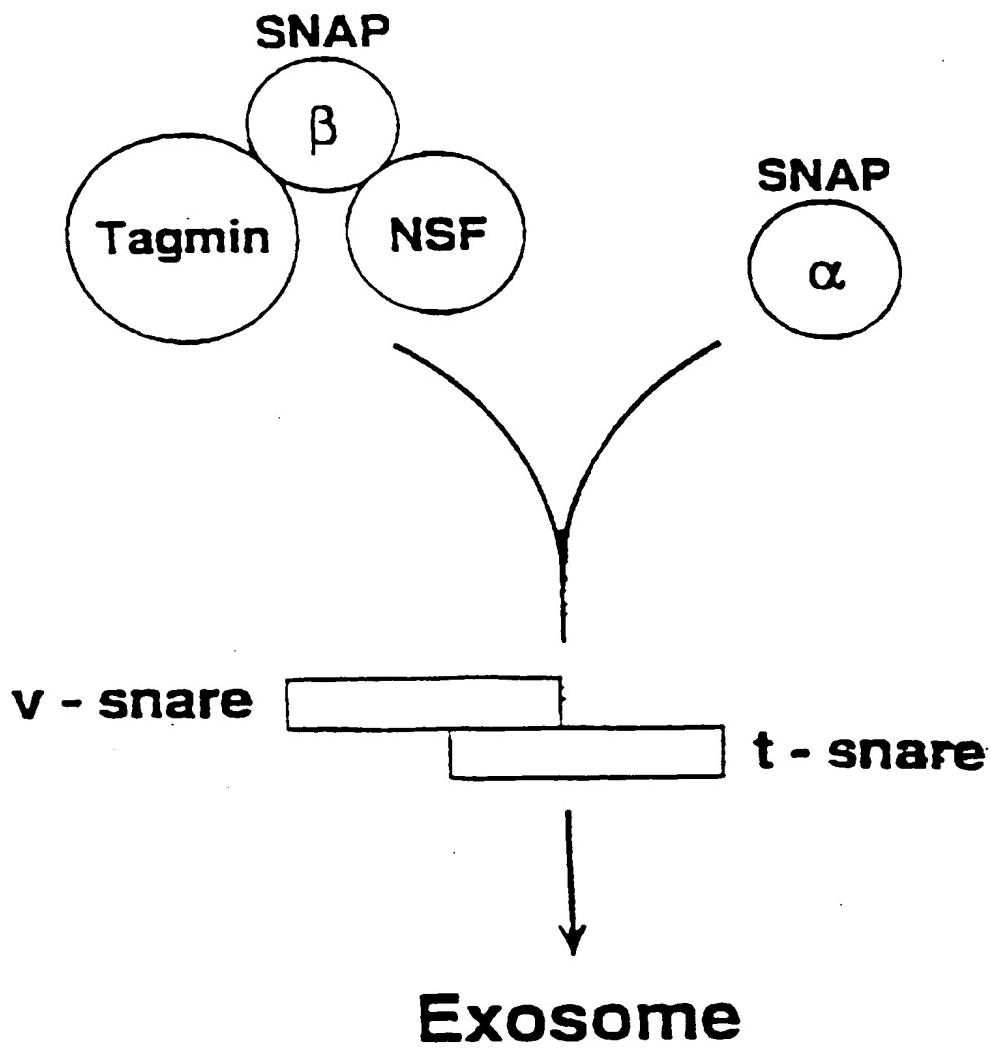
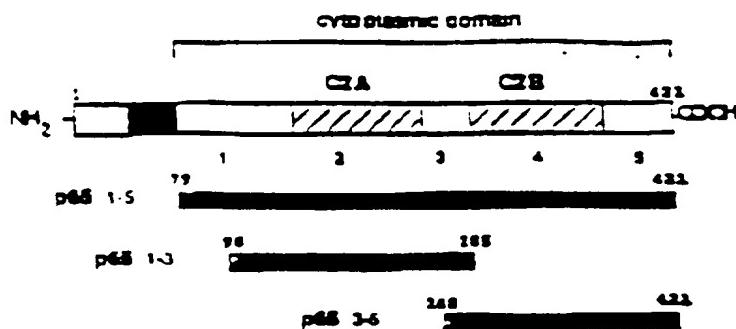
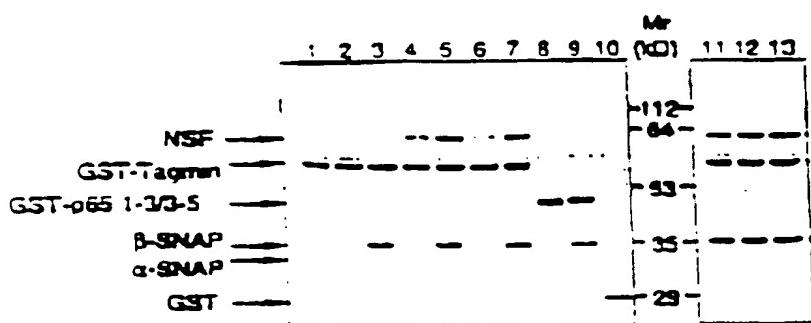


FIG. 1

2 a)



2 b)



GST-Tagmin	- + + + ; + + + -		GST-Tagmin
GST-p65 1-3	- - - - - + + -		B-SNAP
GST-p65 3-5	- - - - - + + -		NSF
GST	- - - - - - - +		Mg ↔
α-SNAP	- + - + + - + -		ATP_{7S}
β-SNAP	- - - + - + + +		ATP
NSF	- - - + + + + -		Ca ↔

2c)

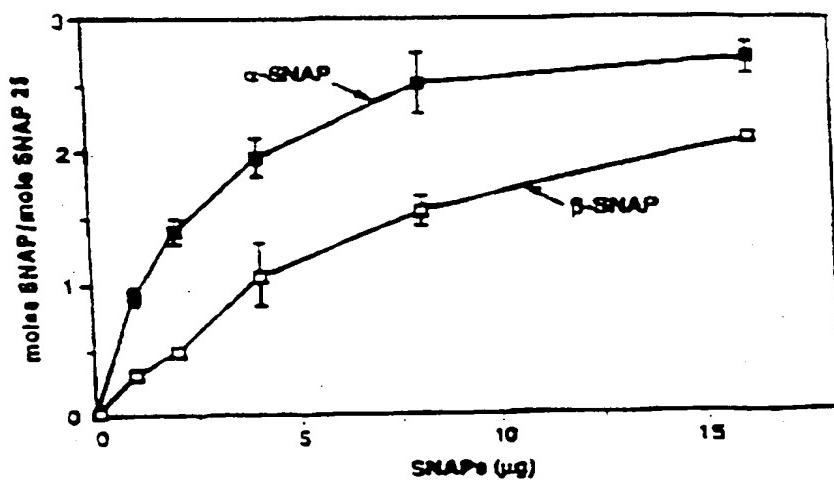


FIG. 2

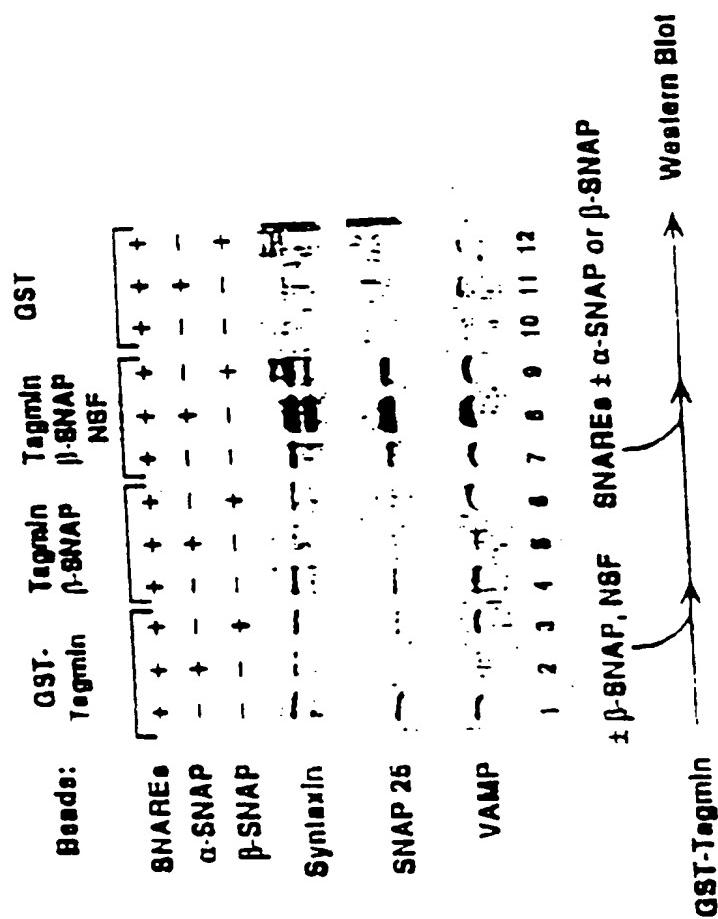


FIG. 3

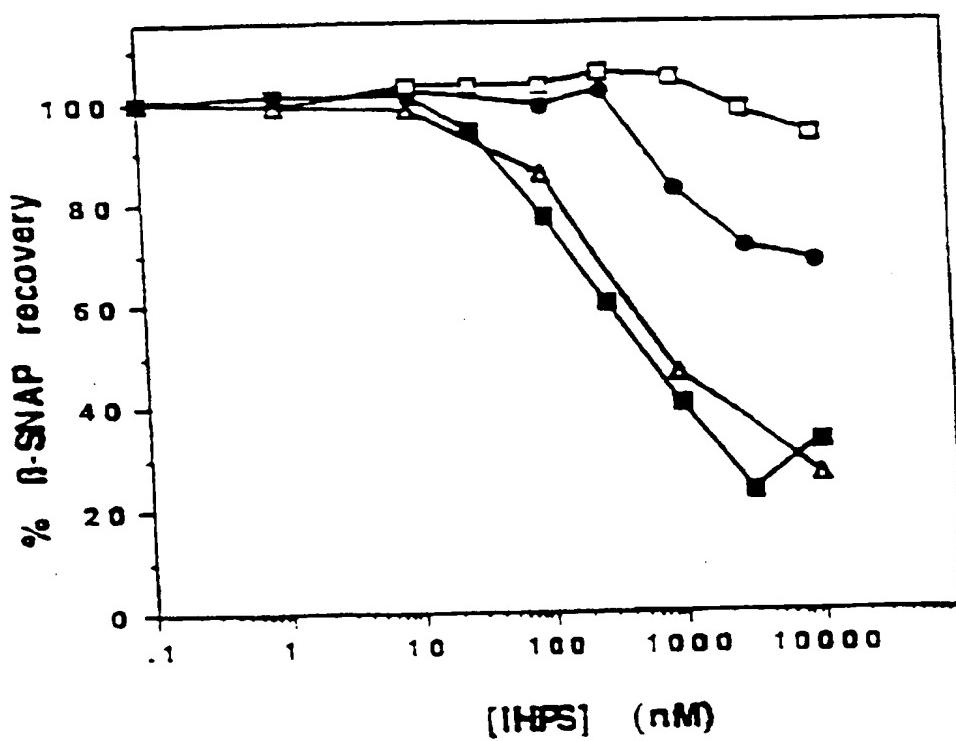


FIG. 4

5/13

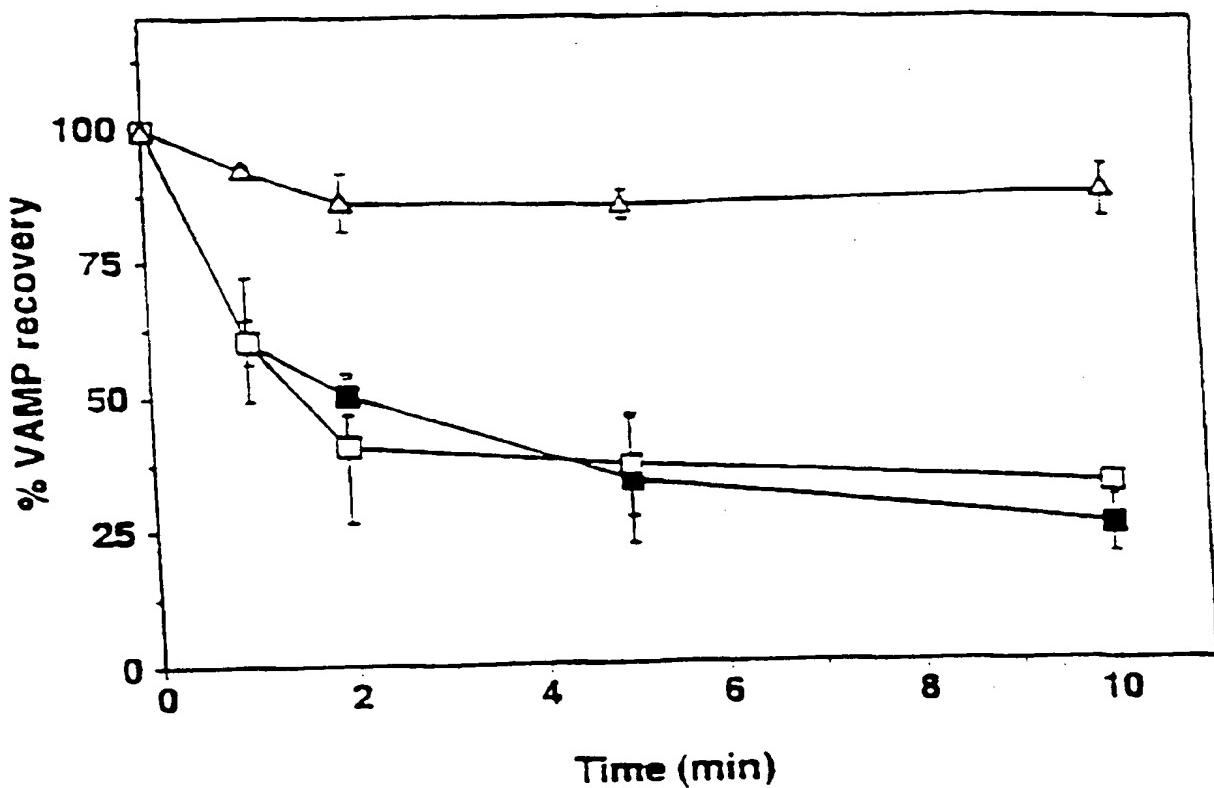


FIG. 5

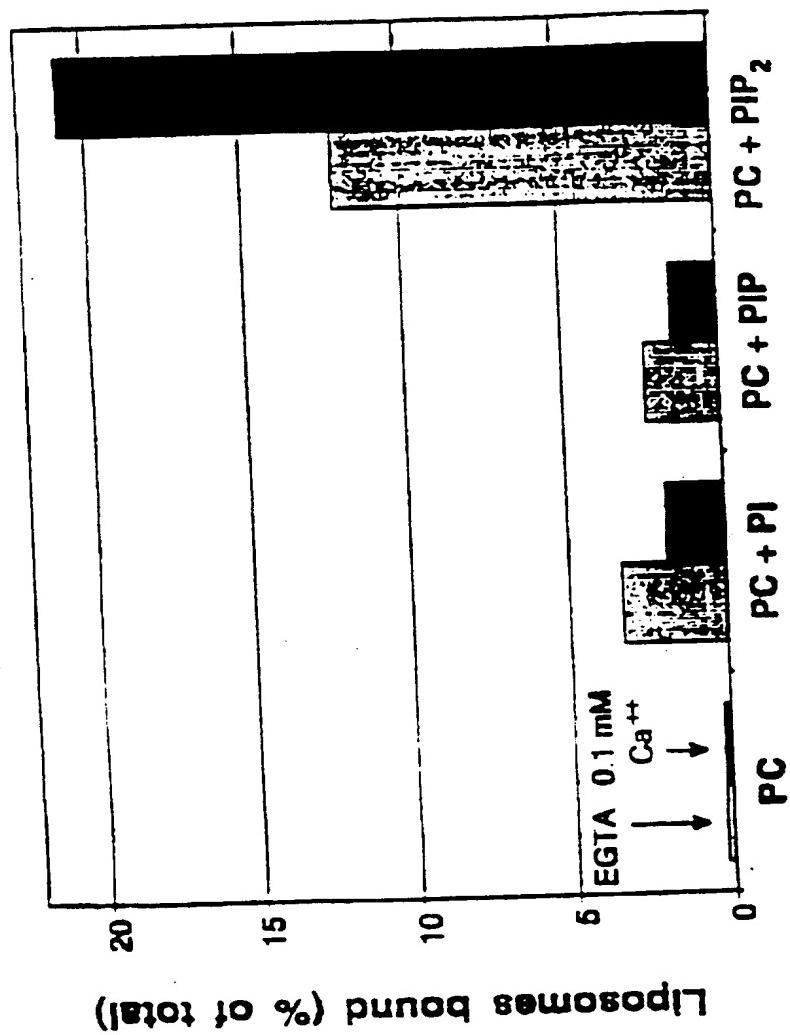


FIG. 6

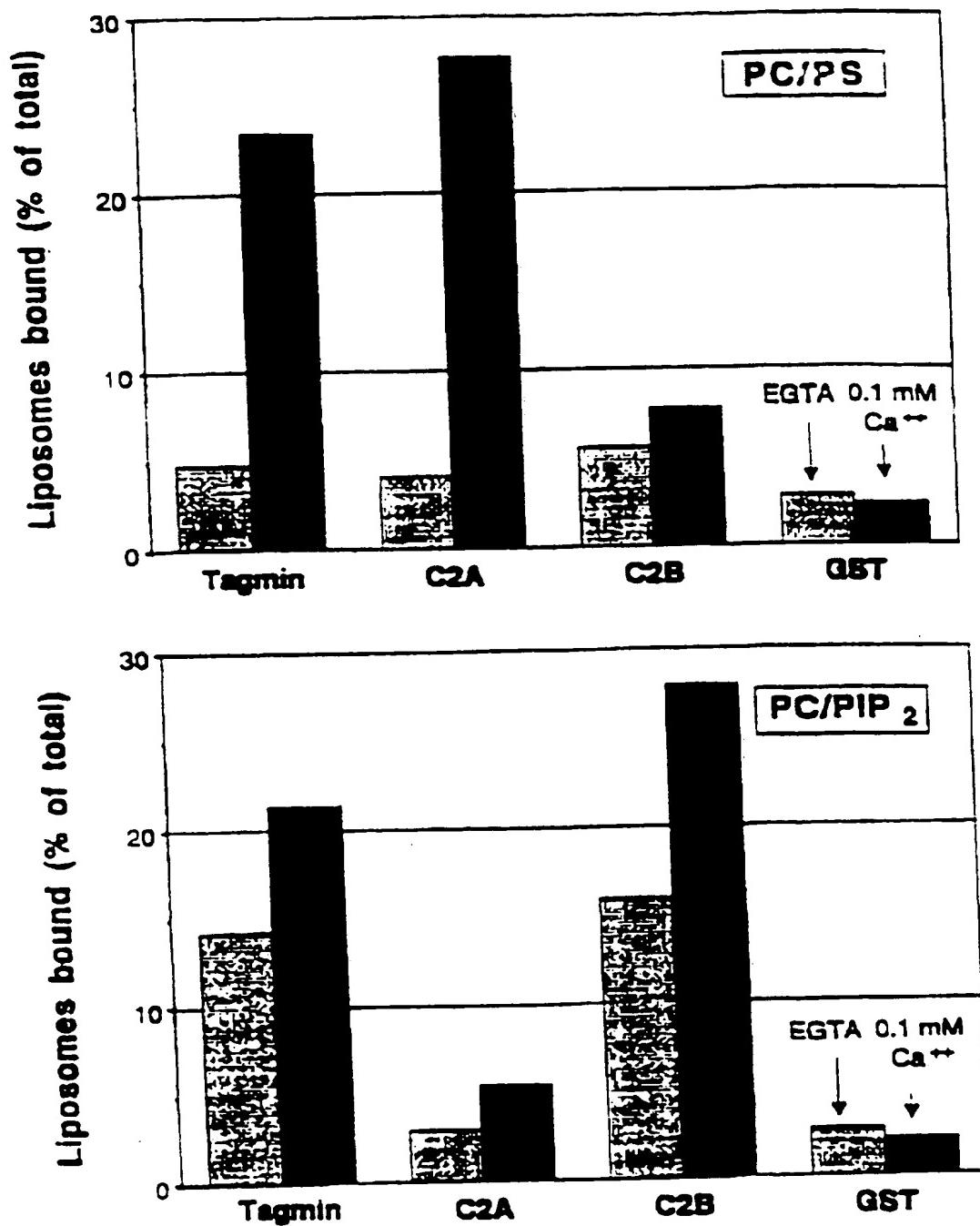


FIG. 7

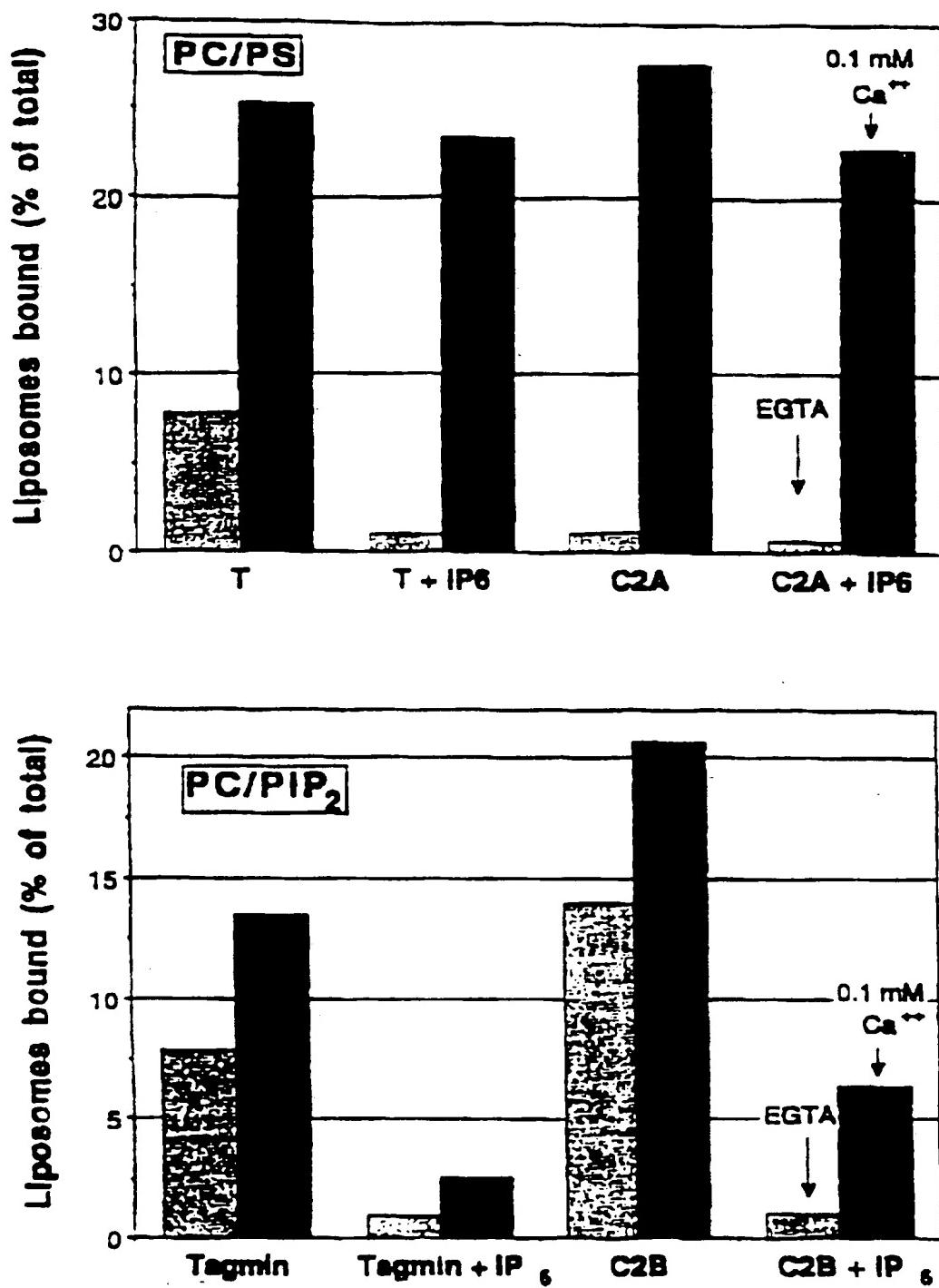
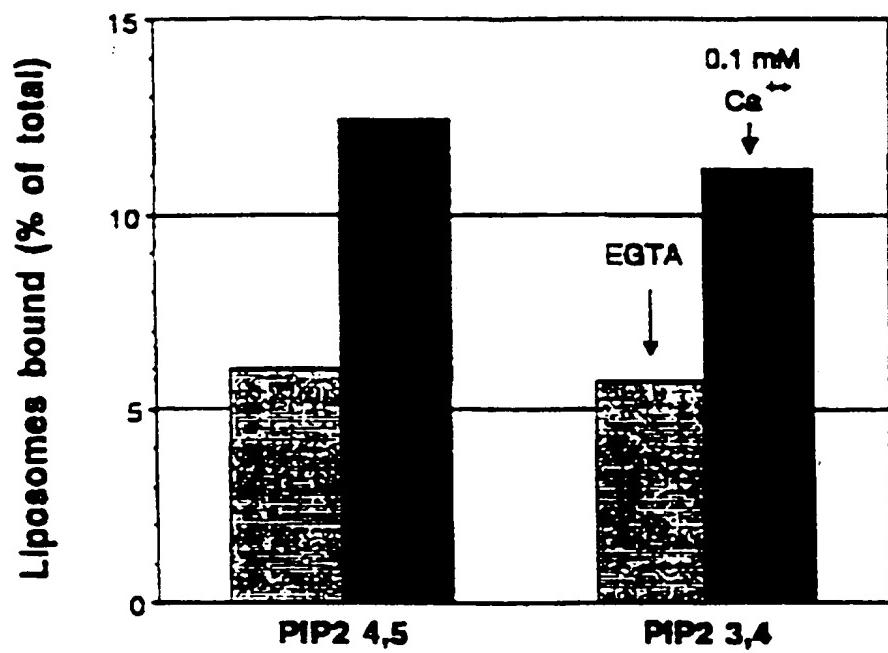
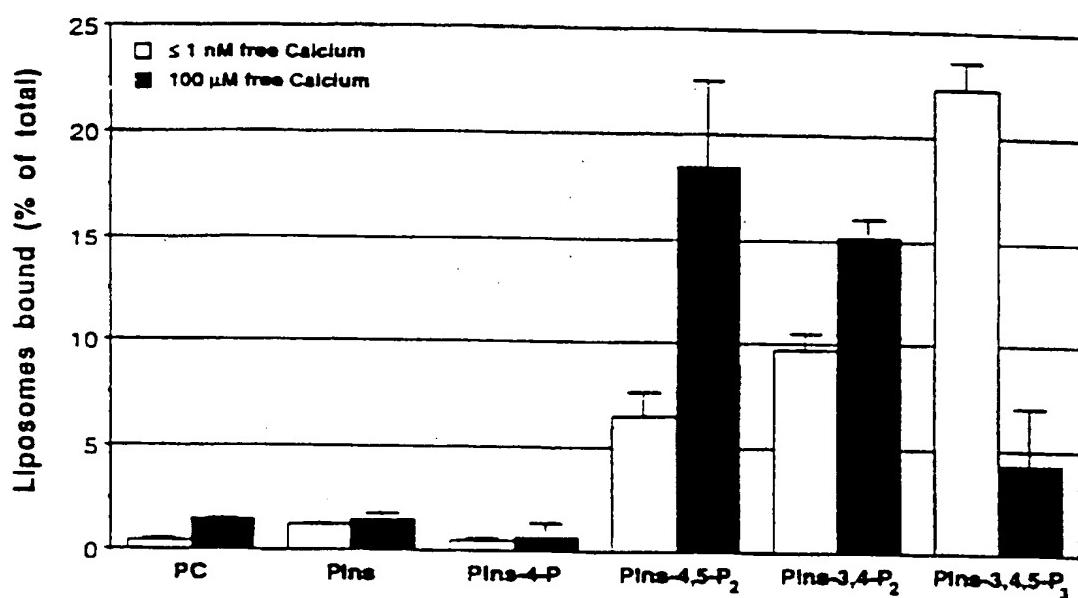


FIG. 8

**FIG. 9**

10/13

**FIG. 10**

11/13

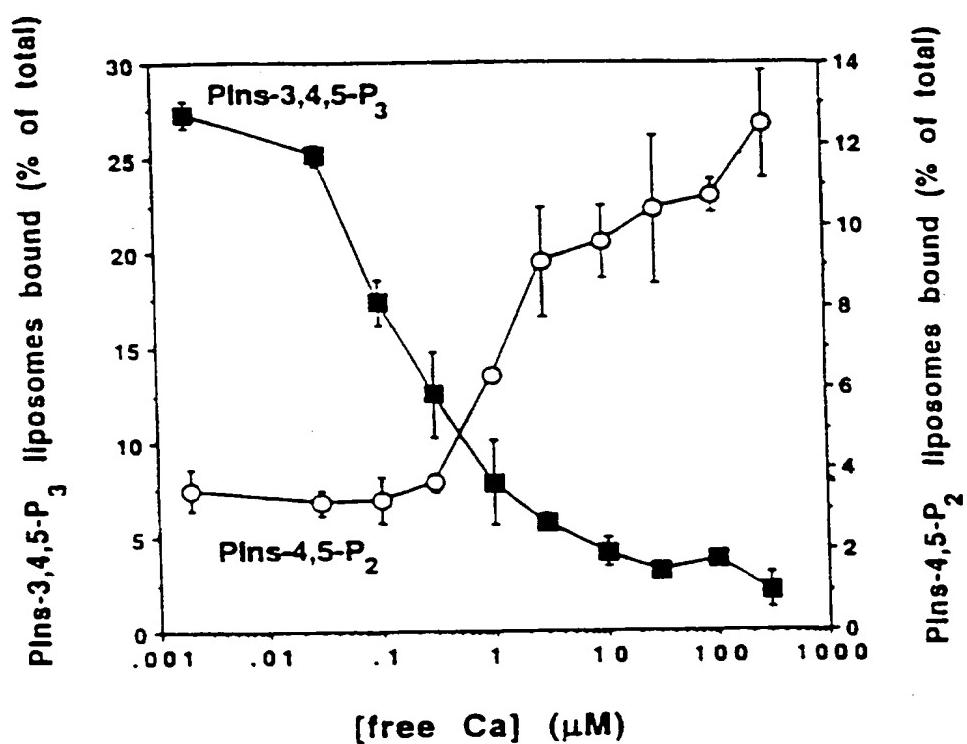
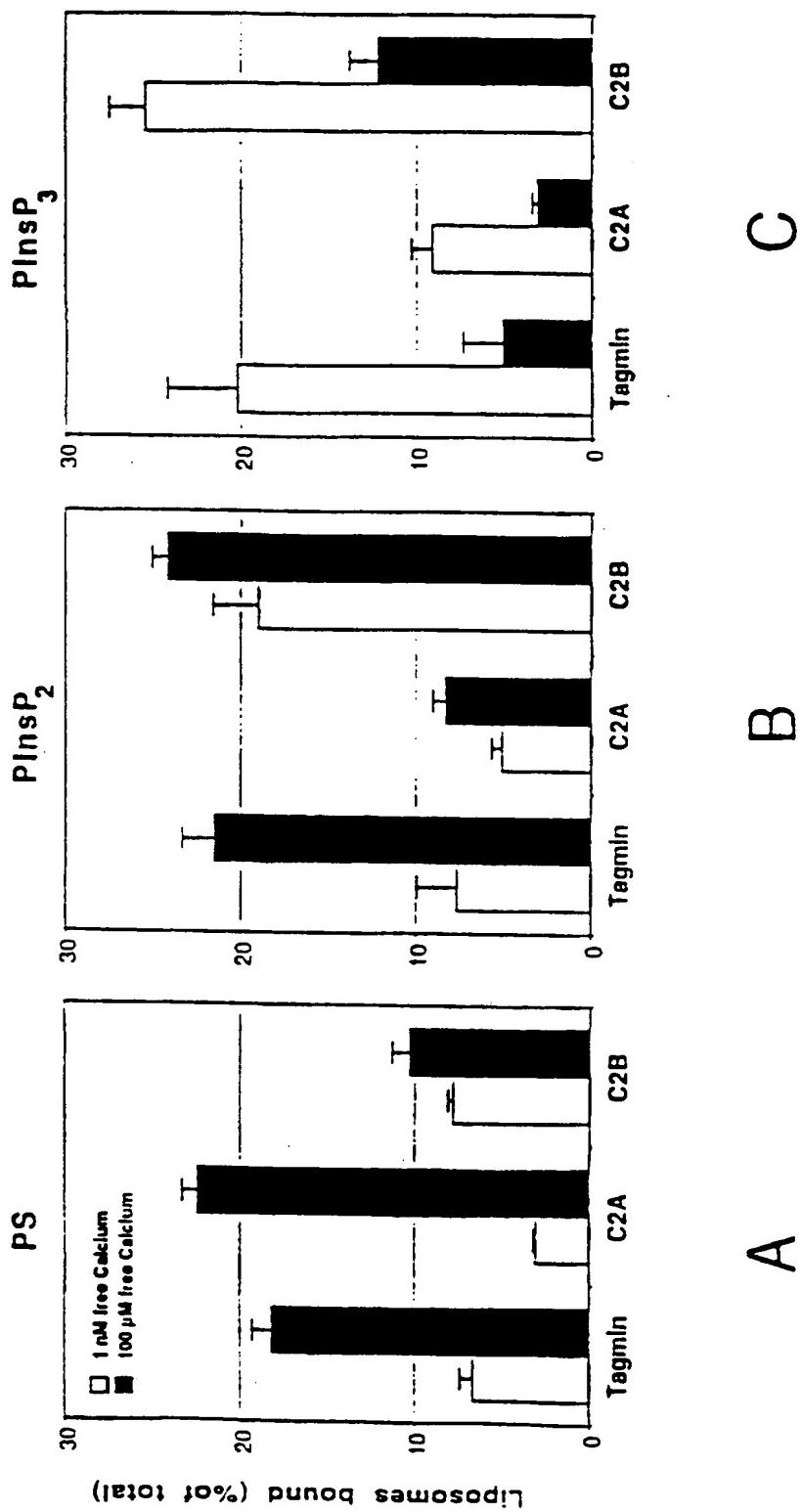
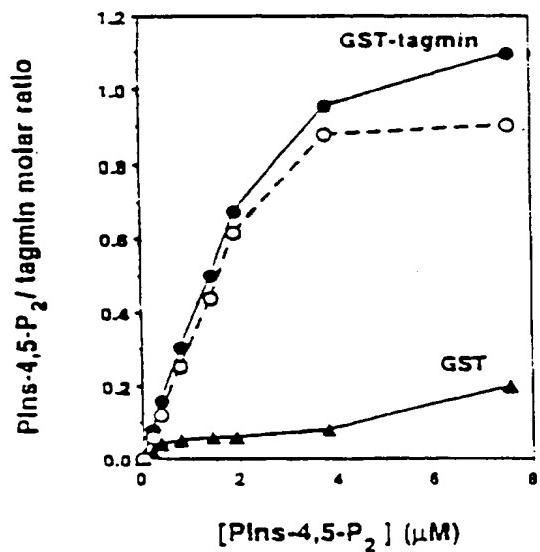
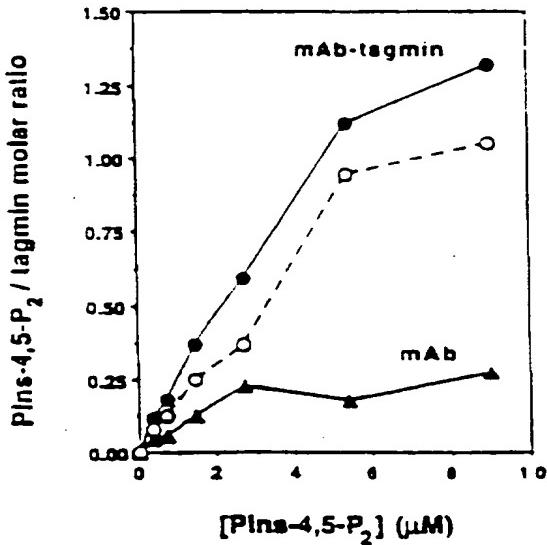
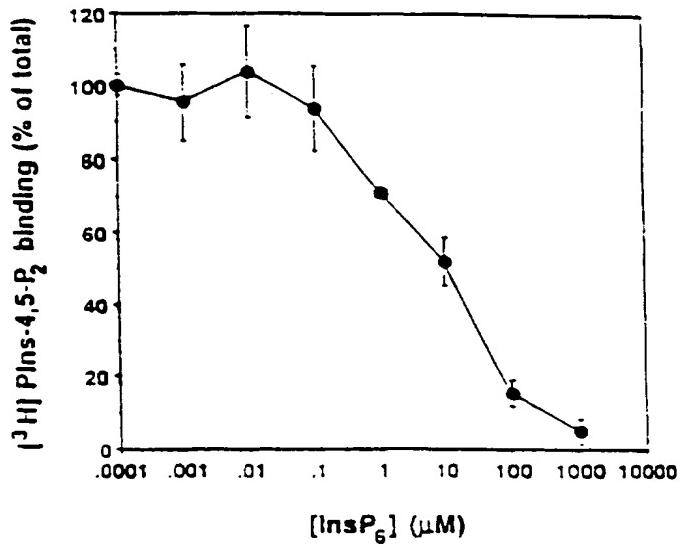
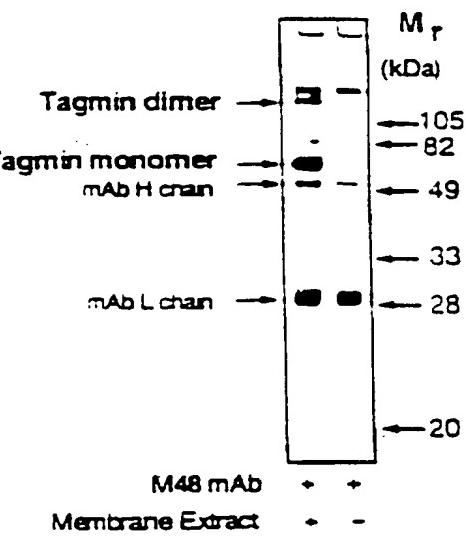


FIG. 11



A**B****C****D****FIG. 13**

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/19661

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/47 G01N33/68 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CELL 75 (3). 1993. 409-418. ISSN: 0092-8674, XP002029580 SOLLNER T ET AL: "A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion." cited in the application see the whole document ---	35,37
X	NATURE, vol. 375, 22 June 1995, pages 645-653, XP002029581 T. SÜDHOFF: "The synaptic vesicle cycle: a cascade of protein-protein interactions" cited in the application see figure 4 ---	35,37



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

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Date of the actual completion of the international search 16 April 1997	Date of mailing of the international search report 25. 04. 97
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Name and mailing address of the ISA
European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl.
Fax (+ 31-70) 340-3016

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INTERNATIONAL SEARCH REPORT

International Application No

PC, US 96/19661

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NEURON, vol. 12, April 1994, pages 909-920, XP000670321 A. DIANTONIO AND T. SCHWARZ: "The effect on synaptic physiology of synatotagmin mutations in Drosophila" cited in the application see page 918, left-hand column, paragraph 2 ---	35,37
X	EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL 14 (19). 1995. 4705-4713. ISSN: 0261-4189, XP002029582 PELLEGRINI L L ET AL: "Clostridial neurotoxins compromise the stability of a low energy SNARE complex mediating NSF activation of synaptic vesicle fusion." see figure 7 ---	35,37
Y	FEBS LETT. (1994), 347(1), 55-8 CODEN: FEBLAL;ISSN: 0014-5793, 1994, XP002029583 PUESCHEL, ANDREAS W. ET AL: "The N-ethylmaleimide-sensitive fusion protein (NSF) is preferentially expressed in the nervous system" see page 57, left-hand column, paragraph 1 ---	1,17
A	NATURE (LONDON) (1993), 362(6418), 353-5 CODEN: NATUAS;ISSN: 0028-0836, 1993, XP002029584 WHITEHEART, SIDNEY W. ET AL: "SNAP family of NSF attachment proteins includes a brain-specific isoform" see page 355, right-hand column, last paragraph ---	
X	NEURON, vol. 13, December 1994, pages 1281-1291, XP000670323 B. ULLRICH ET AL: "Functional properties of multiple synaptotagmins in brain" cited in the application see abstract see page 1289, left-hand column, paragraph 3; figure 5 ---	35,37
Y	---	1,17
	-/-	

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/19661

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, December 1994, WASHINGTON US, pages 12990-12993, XP002029585 R. LLINAS ET AL: "The inositol high-polyphosphate series blocks synaptic transmission by preventing vesicular fusion" cited in the application see the whole document ---	1,17
Y	NATURE, vol. 374, 9 March 1995, pages 173-177, XP002029586 J. HAY ET AL: "ATP-dependent inosite phosphorylation required for Ca ²⁺ -activated secretion" see the whole document ---	1,17
P,X	NATURE, vol. 378, 14 December 1995, pages 733-736, XP002029587 G. SCHIAVO ET AL: "A possible docking and fusion particle for synaptic transmission" cited in the application see the whole document ---	1-16, 18-38
P,X	PROC. NATL. ACAD. SCI. U. S. A. (1996), 93(23), 13327-13332 CODEN: PNASA6; ISSN: 0027-8424, 1996, XP002029588 SCHIAVO, GIAMPIETRO ET AL: "Calcium-dependent switching of the specificity of phosphoinositide binding to synaptotagmin" see the whole document -----	1,17,25, 32-38

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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 14/47, G01N 33/68, A61K 38/17		A1	(11) International Publication Number: WO 97/21729 (43) International Publication Date: 19 June 1997 (19.06.97)
(21) International Application Number: PCT/US96/19661 (22) International Filing Date: 12 December 1996 (12.12.96)		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(30) Priority Data: 60/008,596 13 December 1995 (13.12.95) US 60/030,867 12 November 1996 (12.11.96) US 08/764,786 12 December 1996 (12.12.96) US		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(71) Applicant: SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH (US/US); 1275 York Avenue, New York, NY 10021 (US). (72) Inventors: SCHIAVO, Giampietro; Via L. Cadorna, 16, I-35121 Abano T. Padua (IT). ROTHMAN, James, E.; 402 East 64th Street, New York, NY 10021 (US). SÖLLNER, Thomas, H.; 83 Stratford Avenue, White Plains, NY 10605 (US). (74) Agent: SONNENFELD, Kenneth, H.; Morgan & Finnegan, L.L.P., 345 Park Avenue, New York, NY 10154 (US).			
(54) Title: METHODS AND COMPOSITIONS FOR MODULATING VESICLE RELEASE			
(57) Abstract			
<p>This invention provides methods and compositions useful for modulating synaptic transmission. The compositions of this invention are complexes comprised of various combinations of proteins, such as tagmin, β-SNAP, NSF, α-SNAP, and SNAREs involved in docking and fusion of synaptic vesicles to membranes. In addition, this invention relates to complexes of tagmin and special phospholipids (phosphatidyl inositol) which are a component of membranes involved in the secretory process. These complexes are useful for identifying other substances which may modulate synaptic transmission.</p>			

* (Referred to in PCT Gazette No. 43/1997, Section II)

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(51) International Patent Classification ⁶ : C07K 14/47, G01N 33/68, A61K 38/17		A1	(11) International Publication Number: WO 97/21729
			(43) International Publication Date: 19 June 1997 (19.06.97)
(21) International Application Number: PCT/US96/19661		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).	
(22) International Filing Date: 12 December 1996 (12.12.96)			
(30) Priority Data:			
60/008,596	13 December 1995 (13.12.95)	US	
60/030,867	12 November 1996 (12.11.96)	US	
08/764,786	12 December 1996 (12.12.96)	US	
(71) Applicant: SLOAN-KETTERING INSTITUTE FOR CAN-CER RESEARCH [US/US]; 1275 York Avenue, New York, NY 10021 (US).		Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>	
(72) Inventors: SCHIAVO, Giampietro; Via L. Cadorna, 16, I-35121 Abano T. Padua (IT). ROTHMAN, James, E.; 402 East 64th Street, New York, NY 10021 (US). SÖLLNER, Thomas, H.; 83 Stratford Avenue, White Plains, NY 10605 (US).			
(74) Agent: SONNENFELD, Kenneth, H.; Morgan & Finnegan, L.L.P., 345 Park Avenue, New York, NY 10154 (US).			

(54) Title: METHODS AND COMPOSITIONS FOR MODULATING VESICLE RELEASE

(57) Abstract

This invention provides methods and compositions useful for modulating synaptic transmission. The compositions of this invention are complexes comprised of various combinations of proteins, such as tagmin, β -SNAP, NSF, α -SNAP, and SNAREs involved in docking and fusion of synaptic vesicles to membranes. In addition, this invention relates to complexes of tagmin and special phospholipids (phosphatidyl inositol) which are a component of membranes involved in the secretory process. These complexes are useful for identifying other substances which may modulate synaptic transmission.

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METHODS AND COMPOSITIONS FOR MODULATING VESICLE RELEASE

This application claims priority to pending United States provisional patent application Serial No. 60/030,867 filed November 12, 1996 and pending United States provisional patent application 60/008,596 filed December 13, 1995, the contents of which are incorporated herein by reference.

FIELD OF THE INVENTION

10 This invention relates to methods and compositions useful for modulating the release of vesicular contents from cells. Generally, this invention relates to modulating the process whereby intracellular vesicles fuse with the cell membrane to cause the release of the vesicular contents. More particularly, this invention relates to modulating the releases of synaptic vesicles from neurons. In addition, this invention relates to compositions and methods useful for modulating secretion of substances produced in cells. This invention 15 also relates to methods and compositions useful for screening substances which modulate vesicle release.

20

BACKGROUND OF THE INVENTION

25 Vesicle release from cells, particularly neurons, is a complex process requiring the proper function of various components of the cytoskeleton and soluble proteins. Appropriate ionic flux and response to such flux is also an important aspect of vesicle release. These molecular components and fluxes function together to 30 control the release of neurotransmitter from neurons by providing for calcium mediated fusion of neurotransmitter containing vesicles to the synaptic membrane of the presynaptic neuron. Molecular aspects of synaptic vesicle 35

- 2 -

release have recently been reviewed in Burns et al., Cell 83:187-194 (1995) and Südhof, Nature, 375:645-653 (1995). Exocytosis of transport vesicles has been described in other cells and organisms including yeast. Wilson D.W. et al., Nature, 339:355-359 (1989).

5 Various proteins have been reported to be involved in synaptic vesicle release including: N-ethylmaleimide-sensitive fusion protein (NSF)⁵, synaptotagmin (p65 or tagmin)^{2,8}, soluble NSF attachment proteins (alpha, beta and gamma SNAP) and SNAP receptors (SNAREs)³. Although these proteins have been associated with release of synaptic vesicles their roles and mechanisms of action are still unclear.

10
15 Synaptic vesicle release is believed to result from at least a three step process following the appropriate electrical stimulus. In the first process, docking, the synaptic vesicle is mobilized from a pool of filled vesicles and binds to the presynaptic plasma membrane at active sites. After docking, a slow ATP hydrolysis dependent step termed "vesicle priming" occurs.

20 This priming process makes exocytotic synaptic vesicles competent for the third step, fast calcium dependent fusion. The membranes of the synaptic vesicle and plasma membrane fuse in the last step releasing the vesicle contents into the synaptic cleft. Unfortunately, little

25 has been known about these three steps and the specific proteins involved affording little opportunity for intervention with specific drugs. As recently reviewed, "The proteins responsible for docking vesicles at the membrane are not yet clear, but may include the GTP-

30 binding protein Rab3 as well as UNC-18 or SNARE proteins (or both) ..." Burns et al., Cell, 83:187-194, 187 (1995).

35 A number of proteins have been implicated in the rapid (msec) calcium-controlled release of transmitters at nerve endings^{1,2}, including the general fusion proteins α -SNAP (soluble NSF attachment protein)^{3,4} and NSF (NEM-

- 3 -

sensitive fusion protein)^{3,5,6}, the synaptic SNARE (SNAP receptor)^{3,7}, and the calcium-binding protein synaptotagmin², which likely functions as calcium sensor in exocytosis⁸. In addition, an isoform of α -SNAP termed β -SNAP (83% identical to α -SNAP) is highly expressed in brain⁹; no special role for β -SNAP has yet been reported. It has been unclear how, or if, these separate threads of understanding are entwined to account for synaptic transmission.

Recently, Mikoshiba and coworkers²⁰ have found
that the C2B domain of tagmin binds polyphosphoinositols,
specifically InsP_4 , InsP_5 and InsP_6 , but not InsP_1 .

SUMMARY OF THE INVENTION

15 The present invention relates to methods and compositions useful for identifying substances capable of modulating synaptic transmission. In particular, a protein complex has been identified which is involved in docking of synaptic vesicles to synaptic membranes in connection with synaptic transmission. This complex is comprised of three proteins β -SNAP, tagmin, and NSF and the complex, according to this invention, may be used as a target for substances such as drugs which modulate synaptic transmission. Intermediate complexes comprising β -SNAP and tagmin bind NSF and may also be a target for drugs which modulate synaptic transmission. Another intermediate comprising tagmin, β -SNAP, and NSF binds α -SNAP to form a quaternary complex which is capable of binding to SNARES (syntaxin, VAMP and SNAP25). An aspect of this invention therefore is a cell free protein complex comprising a protein comprising amino acid sequences of β -SNAP sufficient to bind to tagmin and a protein comprising an amino acid sequence of tagmin capable of binding β -SNAP. Another aspect of this invention is a cell free complex comprising a protein comprising amino acid

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- 4 -

sequences of β -SNAP sufficient to bind to tagmin, an amino acid sequence of tagmin capable of binding β -SNAP and NSF protein. Still another complex is the tagmin/ β -SNAP/NSF complex further comprising α -SNAP and, optionally the SNAREs. Complexes comprising tagmin and phosphatidylinositol bisphosphate ("PIns-P₂") and phosphatidylinositol trisphosphate ("PIns-P₃") are another aspect of this invention.

Another embodiment of this invention is the use of the formation of the cell free β -SNAP/tagmin, β -SNAP/tagmin/NSF, or β -SNAP/tagmin/NSF/ α -SNAP or β -SNAP/tagmin/NSF/ α -SNAP/SNARE, or any of the above cited complexes also comprising PIns-P₂ or PIns-P₃ complexes as an assay for the detection of drugs which modulate synaptic transmission. Accordingly, this invention also relates to assay kits comprising the components to conduct such assays including β -SNAP, tagmin and optionally NSF, α -SNAP, SNARE proteins, PIns-P₂, PIns-P₃, and various reagents for detecting the presence of a protein in the complex. These components should be present in an amount sufficient to detect complex formation in the absence of a test substance.

The method of identifying substances capable of inhibiting synaptic transmission comprises combining an amino acid sequence of β -SNAP capable of binding tagmin and a β -SNAP binding protein comprising an amino acid sequence of tagmin with a test substance under conditions which allow for β -SNAP and the β -SNAP binding protein to form a complex and then detecting the formation of β -SNAP/ β -SNAP binding protein complexes. Optionally, the formation of a triple complex further comprising NSF or a quaternary complex further comprising α -SNAP may be assayed in the presence of a test substance. In addition the complex comprising β -SNAP/tagmin/NSF/ α -SNAP and at least one SNARE protein may also be used in this invention. In either assay, substances which are

- 5 -

identified which reduce or inhibit the formation of any of the β -SNAP/tagmin, β -SNAP/tagmin/NSF, β -SNAP/tagmin/NSF/ α -SNAP, β -SNAP/tagmin/NSF/ α -SNAP/SNARE, tagmin/PIns-P₂, or tagmin/PIns-P₃ complexes may be expected to similarly reduce or inhibit synaptic transmission. Conversely, substances capable of increasing complex formation would be expected to facilitate synaptic transmission.

Because different isoforms of tagmin are localized in different parts of the nervous system including different brain regions (see, Ullrich et al., Neuron, 13:1281-1291 (1994)) another embodiment of this invention includes assays for detecting substances which modulate synaptic transmission which are specific for neurons or regions of the nervous system based on the presence of a particular tagmin isoform. Accordingly, in this embodiment of the invention, assays are conducted using specific isoforms of tagmin which are specific for the region of the nervous system for which synaptic transmission is desired to be modified.

The binding of the β -SNAP/tagmin/NSF complex to α -SNAP and SNAREs are additional sites provided by this invention for modulating docking and thereby synaptic transmission.

This invention also provides methods of modulating synaptic transmission by modulating formation of either the β -SNAP/tagmin, β -SNAP/tagmin/NSF, β -SNAP/tagmin/NSF/ α -SNAP, or β -SNAP/tagmin/NSF/ α -SNAP/SNARE complex or tagmin with any of the above cited complexes with PIns-P₂ or PIns-P₃. By providing substances which are specific for certain isoforms of tagmin, this invention also provides highly specific means of modulating synaptic transmission in vivo. Polyphosphoinositols are a class of compounds (PIns-P₂, PIns-P₃, and analogues) provided by this invention which may be used to modulate docking and synaptic transmission.

- 6 -

The synaptic vesicle membrane protein synaptotagmin (tagmin) is essential for fast, calcium-dependent, neurotransmitter release. The present invention has identified a connection between the observations that polyphosphoinositides are necessary for exocytosis and the finding that the C2B domain of tagmin I binds phosphatidylinositol-4,5-bisphosphate (PIns-4,5-P₂), its isomer phosphatidylinositol-3,4-bisphosphate (PIns-3,4-P₂) and phosphatidylinositol-3,4,5-trisphosphate (PIns-3,4,5-P₃). Calcium ions switch the specificity of this binding from PIns-P₁ (at calcium concentrations found in resting nerve terminals) to PIns-P₂ (at concentration of calcium required for transmitter release). The present invention teaches that tagmin operates as a bimodal calcium sensor, switching bound lipids during exocytosis, which is useful in treatment and maintenance of various physiological processes and disorders associated therewith, such as long-term memory and learning. The present invention can also be used to treat various neurological disorders comprising increasing or decreasing the intracellular calcium levels in a patient.

The ability to specifically modulate synaptic transmission by identifying drugs which are specific for various isoforms of tagmin provides a means for specifically treating various neurological disorders associated with improper synaptic transmission; i.e. anatomically different areas of the nervous system.

Antibodies which are specific for the various complexes are also provided by this invention which may be used for either diagnostic or therapeutic purposes.

An object of this invention is to provide compositions and methods for identifying substances useful for decreasing or inhibiting functional docking or priming of vesicles to plasma membranes.

Another object of this invention is to provide compositions and methods useful for inhibiting synaptic

- 7 -

° transmission mediated through docking of synaptic vesicles to SNARES.

Another object of this invention is to provide substances useful for treating various neurological or psychiatric disorders.

5 Yet another object of the present invention is to treat various neurological and psychiatric disorders by modulating neurotransmitter release by manipulating the intracellular calcium levels in a recipient with such a disorder.

10 Another object of this invention is to provide assay systems and kits useful for identifying such substances.

This invention casts new light on synaptotagmin by affording the recognition that this protein is not only 15 a calcium sensor but also a specialized v-SNARE. Like other v-SNAREs³, synaptotagmin is localized to a transport vesicle, binds to a t-SNARE (syntaxin)¹⁴, is a specialized SNAP receptor (binds β -SNAP, but not α -SNAP), enters a docking and fusion particle, and is released when NSF hydrolyzes ATP. The docking and fusion complexes provided 20 by this invention could serve to link, directly or indirectly, the general process of membrane fusion to calcium entry by attaching a specialized fusion protein (β -SNAP) to a v-SNARE that is also a calcium sensor 25 (synaptotagmin). The polyphosphoinositols InsP₄, InsP₅, and InsP₆ are known to block transmitter release and also block the assembly of the particle by preventing β -SNAP from binding to synaptotagmin.

30 BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1. Schematic representation of the formation of protein complexes involved in vesicle docking.

- 8 -

o **Fig. 2.** Binding specificity of α -SNAP and β -SNAP for SNAREs and to tagmin. A) Schematic representation of synaptotagmin I and its fragments (hatched boxes, C2 domains; solid box, transmembrane region). B) Specific binding of β -SNAP to tagmin and to its carboxy-terminal part and the subsequent recruitment of NSF (left panel). C) α -SNAP and β -SNAP binding to the SNARE complex.

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10 **Fig. 3.** Requirements for the assembly of the specialized docking and fusion particle. Immobilized GST-tagmin/ β -SNAP/NSF complexes and GST were incubated with bovine brain detergent extracts in absence or presence of α -SNAP or β -SNAP. Bound syntaxin 1A and 1B (upper panel), SNAP-25 (middle panel) and VAMP (lower panel) were analyzed by immunodecoration of Western blots.

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20 **Fig. 4.** Effect of polyphosphoinositols (IHPS) on the binding of β -SNAP to tagmin. Immobilized GST-tagmin was incubated with β -SNAP in the presence of different IHPS at increasing concentrations. The results were expressed as percentage of the amount of β -SNAP bound to tagmin in the absence of IHPS. (closed squares, InsP₆; open triangles, InsP₅; closed circle, InsP₄; open squares, InsP₃) and represented the average of three to five independent experiments.

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Fig. 5. Mg^{2+} ATP dependent disassembly of the specialized docking and fusion particle. Immobilized complexes were treated under conditions that allow Mg^{2+} ATP dependent SNARE complex disassembly. This process was monitored by detecting the exposure of VAMP to tetanus toxin following disruption of the SNARE complex (open triangles, samples plus Mg^{2+} ATP γ S; open squares, samples plus Mg^{2+} ATP; closed squares, samples plus Mg^{2+} ATP and Ca^{2+}).

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- 9 -

° **FIG. 6.** Binding specificity of GST-tagmin to liposomes of different compositions. GST-tagmin was incubated with pure 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine liposomes (PC) or PC containing 1% w/w 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-D-myo-inositol (PI) or 5 1% (w/w) 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-D-myo-inositol-4-phosphate (PIns) or 1% (w/w) 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-D-myo-inositol-4,5-bisphosphate (PIns 4,5-P₂) in the presence or absence of 10 100 μM free calcium. Liposome binding was quantified by liquid scintillation counting of the [³H]PC used as tracer and expressed as % of total radioactivity used. The unspecific binding of liposomes to the beads was determined by using pure GST and the results were subtracted from the total binding.

15 **FIG. 7.** The binding of PIns-P₂ on synaptotagmin is localized to the carboxy-terminal domain. GST-tagmin, its amino terminal (C2A) and carboxy-terminal (CB2) domains were incubated with PC and 1,2-dioleoyl-sn-glycero-3-[phospho-L-serine] (PS) (3:1 w/w) (upper panel, Fig. 7A) 20 or with PC liposomes containing 1% (w/w) of PIns-P₂ (lower panel, Fig. 7B). Liposome binding was determined as in Fig. 6.

25 **FIG. 8.** The binding of tagmin and the tagmin C2B domain to PIns-P₂ (Fig. 8B), but not the calcium-dependent binding of C2A to PS (Fig. 8A), is inhibited by inositol 1,2,3,4,5,6-hexakisphosphate. Liposome binding was determined as in Fig. 6.

30 **FIG. 9.** Synaptotagmin binds to both PIns-P₂ isomers with similar efficiency. Liposomes of PC containing 1% (w/w) of phosphatidylinositol-4,5-bisphosphate (PIns 4,5-P₂) or phosphatidylinositol-3,4-bisphosphate (PIns 3,4-P₂) were 35 incubated with GST tagmin in the presence or absence of

- 10 -

° 100 μ M free calcium as described in Fig. 6.

Fig. 10. Synaptotagmin binds specifically to PInsPs-containing liposomes in a calcium-dependent manner. GST-tagmin was incubated with liposomes containing PC or PC together with distinct phosphoinositides (as indicated) in the presence or absence of calcium ions (shaded bars, 2 mM EGTA; filled bars, 100 μ M free Ca^{2+}). Lipid binding was quantified by liquid scintillation counting of the radioactive PC used as tracer and expressed as % of total radioactivity used.

Fig. 11. Calcium dependency of PIns-4,5-P₂ and PIns-3,4,5-P₃ binding to tagmin. GST-tagmin beads were simultaneously incubated with two populations of PC liposomes either containing PIns-4,5-P₂ or PIns-3,4,5-P₃, at variable Ca^{2+} concentrations (open circles, PIns-4,5-P₂; closed squares, PIns-3,4,5-P₃) .

Fig. 12. The PInsPs binding site on tagmin is localized to the C2B domain. GST fusion proteins containing tagmin or its amino terminal (C2A; aa. 96-265; 10 μ g) or the carboxy-terminal (C2B; aa. 248-421; 10 μ g) domains were incubated with liposomes containing PC and either 25% (w/w) 1,2-dioleoy-sn-glycero-3-phospho-L-serine (PS) (panel 12A) or 1% (w/w) PIns-4,5-P₂ (panel 12B) or 1% (w/w) PIns-3,4,5-P₃ (panel 12C) (shaded bars, 2 mM EGTA; filled bars, 100 μ M free Ca^{2+}).

Fig. 13. The binding of PIns-4,5-P₂ to recombinant and native tagmin is saturable and is competed by InsP₆. A) GST-tagmin (filled circles) or GST alone (filled triangle) were incubated at increasing concentration of radioactive PIns-4,5-P₂ in detergent micelles in the presence of 2 mM EGTA.

- 11 -

DETAILED DESCRIPTION OF THE INVENTION

This invention relates to the process of synaptic release. More specifically, this invention relates to methods and compositions useful for modulating docking of synaptic vesicles to membranes based on the formation of specific protein complexes which, according to the invention, mediate functional docking of synaptic vesicles. The mechanism identified for docking may be modelled in vitro in cell free systems according to the invention by detecting the formation of various protein complexes.

The various aspects of docking which may be modelled in vitro include the initial binding of tagmin and β -SNAP to form a bimolecular complex which further binds NSF to form a trimolecular complex. This trimolecular complex then binds α -SNAP and α -SNAP receptor (SNAREs) to accomplish docking (Figure 1). Inhibition of any of these complexes would decrease or inhibit synaptic transmission. Conversely, enhancement of complex formation would be expected to facilitate synaptic transmission.

The compositions of this invention are cell free protein complexes comprised of proteins involved in binding of synaptic vesicles to plasma membrane associated with synaptic transmission. The complexes of this invention comprise tagmin and β -SNAP (Figure 2) and may optionally contain one or more additional components such as, for example NSF, α -SNAP, SNAREs, (Figure 3) polyphosphoinositols such as, for example, inositol 1,2,3,4,5,6 hexakis phosphate (IP₆) and inositol 1,3,4,5,6 pentakisphosphate (IP₅) or polyphosphoinositides, such as, for example, phosphatidylinositol-4,5-bisphosphate (PIns-4,5-P₂) or phosphatidylinositol-3,4,5 trisphosphate (PIns-3,4,5-P₃). One such complex of the invention comprises tagmin (also referred to in the art as synaptotagmin or

- 12 -

o p65) and β -SNAP (soluble NSF attachment protein). Yet another complex of this invention comprises tagmin and PIns-P₂, with or without β -SNAP or tagmin and PIns-P₃, with or without β -SNAP. Another such complex of the invention comprises tagmin, β -SNAP and NSF (N-ethylmaleimide-sensitive fusion protein). Still another complex of the invention comprises a quaternary complex of β -SNAP/tagmin/NSF and α -SNAP. Another complex comprises the quaternary complex bound to one or more SNARE proteins. The formation of these complexes in vitro provide the basis for assays useful for identifying substances which modulate synaptic transmission and would therefore be useful as therapeutics or diagnostic reagents.

As used in this invention, "tagmin" refers to any amino acid sequence which comprises at least, the portion of the carboxy terminal region of tagmin which binds β -SNAP. Other amino acid sequences which are sufficiently homologous to this portion of the amino acid sequence of tagmin and which retain the ability to bind β -SNAP are also suitable for use in this invention.

Native tagmin isolated from synaptic vesicles (as, for example, the isolation of tagmin I as described in Südhof et al., Neuron 2:1475-1481 (1989)), or recombinant tagmin obtained from cells which have been transformed with a gene encoding tagmin, or a fragment thereof, are suitable for use in this invention. Recombinant tagmin, or fragments thereof, may be expressed either directly or as a fusion protein attached to additional amino acids. Such methods of expressing protein are known in the art and are also described herein in the examples. cDNA cloning and the construction of vectors for expression of tagmins or specific amino acid sequences of various tagmins fused to glutathione S-transferase (GST) has previously been described in Ullrich et al., Neuron, 13:1281-1291 (1994) and Li et al., Nature, 375:594-599 (1995).

Tagmin exists in different isoforms which are

- 13 -

° distributed throughout the nervous system. Ullrich et al., Neuron 13:1281-1291 (1994). Because certain isoforms are specific for particular regions, it is therefore possible, through this invention, to identify substances which modulate synaptic transmission in specific regions of the nervous system. For example, in the rat and mouse brain, isoforms of tagmin have been described, synaptotagmins I to VIII. Synaptotagmins I to V are localized at the level of the nervous system. Synaptotagmins I and II are the two more abundant isoforms and are generally distributed in the rostral and caudal brain regions respectively. Synaptotagmins III and IV are reported to be coexpressed in neurons expressing synaptotagmins I and/or II, but also exhibit differences in regional distribution. Whereas synaptotagmin IV is reported to be distributed throughout the nervous system, synaptotagmin III exhibits pronounced regional differences. Synaptotagmin III is expressed at appreciable levels in the cortex, hippocampus and olfactory bulb and at higher levels in neurons of the basal ganglia and thalamus. Other regions exhibiting appreciable expression of synaptotagmin III include neurons of the midbrain, caudal brainstem, motor nuclei magnocellular part of the red nucleus and the gigantocellular neurons of the reticular formation. Heterogeneity of synaptotagmin III distribution is also observed in the spinal cord where motoneurons exhibited high levels of expression. A more detailed description of the distribution of synaptotagmins is present in Ullrich et al., Neuron, 13:1281-1291 (1994) which is incorporated herein by reference. Based on this regional distribution of tagmins, the present invention provides a means of selectively modulating synaptic transmission by targeting substances for specific tagmin isoforms.

In addition to using the entire tagmin protein, smaller amino acid sequences of tagmin may be used which retain the ability to bind β -SNAP. Specific amino acids

- 14 -

may be substituted provided the ability to bind β -SNAP is retained. Based on results described herein, the preferred region of tagmin for use in this invention is the carboxy terminal (C2B) region as this is the region which binds β -SNAP. For synaptotagmin I, fragments comprising the entire cytoplasmic domain, the C2B domain, amino acids 79-421, or 248-421 retain the ability to bind β -SNAP and are preferred. More preferred is the amino acid region of 248-421. Preferred amino acid sequence for other tagmin isoforms are as follows: tagmin II, amino acid residues about 249 to 422; tagmin III, amino acid residues about 405-588; tagmin IV, amino acid residues about 263-425; tagmin V, amino acid residues about 116 to 279; tagmin VI, amino acid residues about 338-511; tagmin VII, amino acid residues about 242 to 403; and tagmin, VIII amino acid residues 177 to 255. The amino acid sequences recited above are based on rat tagmin isoforms but would substantially correspond to the tagmin isoforms of other species as well. The amino acid sequences of the proteins for use in this invention may be modified by substituting amino acids provided they retain the binding characteristics necessary to assess complex formation. In addition, the length of the amino acid fragments described above may be varied again provided binding to β -SNAP remains intact.

β -SNAP is a brain specific 34Kd NSF binding protein capable of binding to SNAP receptors. The amino acid sequence of bovine β -SNAP is known. Whiteheart et al., Nature, 362:353-355 (1993) which is incorporated herein by reference. β -SNAP may be used in accordance with this invention as the full length protein. A peptide corresponding to amino acids 25 to 45 of β -SNAP inhibits the interaction between β -SNAP and tagmin and for this reason, the amino terminal end of β -SNAP is thought to interact directly with tagmin. This preferred peptide could be used to modulate the β -SNAP/tagmin interaction

- 15 -

sequence for use in this invention. Like tagmin, native β -SNAP or a recombinant form may be used.

NSF is a protein which has been described by Wilson et al., Nature, 339:355-359 (1989). The amino acid sequences of NSF from various different animal species are known. Methods of recombinantly expressing NSF have also been described. Wilson et al., Nature, 339:355-359 (1989) and Wilson and Rothman, Meth. Enzymol., 219:309-318 (1990). For use in this invention, NSF may be expressed recombinantly using any of the expression systems available to those in the art including, for example E. coli. In addition, native NSF purified from natural sources may be used.

For use in this invention, the species from which the various proteins are derived is not significant as there is substantial homology across species and complexes can be formed using proteins from different species. For example in the examples described herein, β -SNAP and α -SNAP amino acid sequences are bovine, NSF is a hamster sequence, and tagmin is a rat sequence.

The formation in vitro and in vivo of any of the bimolecular (β -SNAP/tagmin; PIns-P₂/tagmin and PIns-P₃/tagmin), trimolecular (β -SNAP/tagmin/NSF), quaternary (β -SNAP/tagmin/NSF/ α -SNAP) or pentameric (β -SNAP/tagmin/NSF/ α -SNAP/SNAREs) complexes of this invention and their hydrolysis by Mg²⁺ ATP (Figure 5) provides a means for identifying substances which would be expected to be useful to modify synaptic transmission. Thus, substances which decrease or inhibit complex formation in vitro are useful as drug leads from which therapeutics may be identified. Such therapeutics are useful for treating or diagnosing various neurological or psychiatric disorders for which decreasing or inhibiting synaptic transmission generally or in specific regions of the nervous system is desirable. Examples of neurological disorders for which decreasing or inhibiting synaptic

- 16 -

transmission would be expected to be therapeutic include epilepsy which is characterized by improper electrical discharge in one or more brain regions; and diseases such as Huntington's, in which pathology is related to the action of an excitatory neurotransmitter. Bipolar manic-depressive disorder which is currently treated with lithium is another disease for which decreasing synaptic transmission through inhibition of complex formation would be expected to be therapeutic. Pathologies of the neuromuscular junction or pathologies associated with motor neuron function (such as dystonias) may also be related to the physiology of these complexes.

The assays of this invention for identifying substances which modulate synaptic transmission may be carried out using various protocols known to those in the art for measuring binding of molecules to proteins. For the purposes of this invention, any assay capable of detecting relative amounts of complex formation in the presence and absence of test substances is suitable. In one embodiment, one of the complex components is immobilized on a solid support, e.g., agarose beads or immobilized antibodies which is then combined in an appropriate buffer with the other components of the complex in the presence and absence of test substance. The solid support is then separated from the unbound components and the formation of the complex is assessed by detecting the presence of the various components bound to the solid support. After separation of the complex from the free components, the presence of the components in the complex may be detected either by direct analysis of the complex bound to the solid support or following dissociation of the complex and analysis of the liberated components. Presence of β -SNAP, tagmin, NSF or α -SNAP in any of the complexes may be detected, for example by using specific antibodies. Any method known in the art for detecting the presence of a specific antibody may be used

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- 17 -

in the assays of this invention including the use of radioactive isotopes, chemiluminescence or enzymatic action. In the preferred mode of carrying out the assays of this invention, the formation of each form of complex (β -SNAP/tagmin; β -SNAP/tagmin/NSF; β -SNAP/tagmin/NSF/ α -SNAP; or β -SNAP/tagmin/NSF/ α -SNAP/SNARE) is tested separately with test substances to determine the effect on complex formation and on the capability to bind PIns-P₂ or PIns-P₃. Alternatively, it may also be desirable to combine all of the components together in one reaction to assess the overall effect.

In addition to identifying substances which influence formation of the binary, tertiary, quaternary, or pentameric complexes, the present invention also identifies the formation of other complexes which are involved in docking of vesicles to plasma membrane to cause vesicle release. According to the model provided by this invention, prior to vesicle release, the tertiary tagmin/ β -SNAP/NSF complex interacts with α -SNAP and is then capable of binding SNAREs to cause vesicle docking. The binding of α -SNAP to the tertiary tagmin/ β -SNAP/NSF complex to form the quaternary tagmin/ β -SNAP/NSF/ α -SNAP provides another site useful for identifying potentially therapeutic substances and for modulating synaptic transmission. Binding of the tagmin/ β -SNAP/NSF/ α -SNAP complex to a SNARE complex, i.e. docking, provides yet another site for identifying substances which may be useful to inhibit synaptic transmission.

Support that the model provided herein for docking of synaptic vesicles to plasma membranes is predictive for identifying substances which modulate synaptic transmission is provided by the effects of polyphosphoinositols (InsP₄, InsP₅ and InsP₆) on complex formation. As shown in Figure 4, these compounds, which have previously been reported to inhibit synaptic release²¹ and bind tagmin²⁰ inhibited in vitro the formation of the

- 18 -

- o cell free tagmin and β -SNAP complexes. Other phosphoinositols besides InsP₄, InsP₅ and InsP₆ and which are identified according to the methods of the present invention are preferred for use in the methods of inhibiting docking and synaptic transmission.

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Preparation of Complex Specific Antibodies

The complexes of this invention are also useful as immunogens to raise antibodies (polyclonal or monoclonal) specific for each form of complex (β -SNAP/tagmin; β -SNAP/tagmin/NSF; β -SNAP/tagmin/NSF/ α -SNAP; and β -SNAP/tagmin/NSF/ α -SNAP/SNARE). Preferably such antibodies are conformation-specific in that they recognize the functional form of the various complexes of this invention. These antibodies may then be used as specific substances for decreasing synaptic release or as diagnostics for detecting the presence of intracellular complexes. Normally the blood brain barrier would prevent passage of peripherally administered antibodies. However, in various neurological disorders, particularly those associated with trauma which may precipitate improper synaptic release (i.e., epilepsy), the blood brain barrier may be more permeable allowing passage of such peripherally administered antibodies. Alternatively, large molecules such as antibodies may be administered directly into spinal fluid or intrathecally.

The requirement of several proteins to effect docking significantly increases the likelihood that one or more neurological diseases results from improper expression or function of one of these proteins leading to improper complex formation. The antibodies which are provided by this invention may be used to analyze tissue for the presence of appropriate complexes.

- 19 -

Binding of tagmin and β -SNAP/tagmin
Complexes to Membrane Phospholipids

Whereas the polyphosphoinositols described above inhibit complex formation, an inositol-containing phospholipid, phosphatidylinositol-4,5-bisphosphate, the isomer phosphatidylinositol-3,4-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate present as minor constituents in cellular membranes are compatible with tagmin/ β -SNAP complexes. As used herein, PIns-P₂ refers to either of the phosphatidyl inositol biphosphates, while PIns-P₃ refers to any of the phosphatidylinositol triphosphates. Other analogs of polyphosphatidylinositol may also be suitable for use with this invention and may vary based on the length of the fatty acid chain, for example C₁₆ to C₂₄ and location and number of double bonds or the degree and position of phosphorylation of the inositol ring, although monophosphates appear inactive.

The present invention also relates to a novel calcium sensor and switch important in regulating the priming step of synaptic neurotransmission. Calcium has been identified as the signal for altering the affinity of various inositol polyphosphates. In the absence of free calcium the naturally occurring phosphatidylinositol bisphosphates, PIns-4,5-P₂ or PIns-3,4-P₂, only moderately increase the interaction of tagmin with the liposomes (Fig. 10A). However, under the same conditions, PIns-3,4,5-P₃ strongly promotes the binding of liposomes to tagmin. Fig. 10. The inclusion of phosphatidylinositol (PIns) or phosphatidylinositol-4-phosphate (PIns-4-P) does not result in binding of liposomes to tagmin, either in the presence (100 μ M) or in the absence of free calcium. Fig. 10A.

Calcium (about 100 μ M) switches the specificity of these interactions. Binding of tagmin to PIns-3,4,5-P₃-containing liposomes is greatly reduced, while tagmin

- 20 -

now binds efficiently to both PIns-4,5-P₂ and PIns-3,4-P₂-containing liposomes. Fig. 10. Qualitatively similar results were obtained by using native tagmin and recombinant tagmin, thus indicating that the conclusions obtained with the GST-tagmin have a general validity

5 regardless of the source of tagmin.

The dependence of PIns-4,5-P₂ and PIns-3,4,5-P₃ binding on the concentration of free calcium is demonstrated by the present invention (see Fig. 11).

Tagmin loses the capacity to bind PIns-3,4,5-P₃ between about 0.1 and 1 μM free calcium. It gains the capacity to bind PIns-4,5-P₂ progressively above about 1 μM free calcium with maximum binding reached only at about 100 μM. The calcium dependency of the switch of specificity is not significantly affected by altering the concentration of PIns-4,5-P₂ or PIns-3,4,5-P₃ in the liposome. The presence of magnesium is preferred to maximize the complete switch. A preferred concentration of magnesium is about 0.5-1.0 mM. This calcium switch is useful in modulating the rate of synaptic neurotransmission and provides a valuable target of substances acting on neurotransmitter release. The identification of this calcium switch provides a therapeutic tool for the treatment of various neurological, psychiatric, memory and learning disorders.

Tagmin contains two C2 domains homologous to the calcium and acidic phospholipid binding domain of protein kinase C.^{29,30} The more amino-terminal C2 domain (C2A) binds PS (25 % w/w) in a calcium-dependent manner with a Kd of 3-6 μM.^{27,28} The more carboxy-terminal C2 domain (C2B) binds InsPP and interacts with both PIns-P₂ and PIns-P₃. While the interaction of the C2B domain with PIns-3,4,5-P₃ is reduced by calcium, as for the entire cytoplasmic domain, the interaction of the C2B domain with PIns-4,5-P₂ is strong even in the absence of calcium, and only weakly enhanced by calcium, implying cooperativity between the two C2 domains. While the C2A domain is known

- 21 -

to bind liposomes containing PS in a calcium dependent manner,^{27, 28} the C2B domain binds PS-containing liposomes weakly and in a calcium independent manner,^{20, 32} further establishing the specificity of the binding to polyphosphoinositides. Fig. 12A.

5 The interaction of both tagmin and C2B with PIns-4,5-P₂ and PIns-3,4,5-P₃-containing liposomes is competitively inhibited by InsP₆, suggesting that all three compounds bind to the same site in the C2B domain. InsP₆ does not affect the calcium-dependent interaction of
10 PS-containing liposomes with tagmin or its C2A domain. In summary, the C2B domain appears to preferentially interact with polyphosphoinositides; however the C2A domain may have some binding activity. For full calcium-sensitivity, it is preferred that the C2A and C2B domains of tagmin be
15 present.

Micellar PIns-P₂ binds to recombinant and native tagmin and is inhibited by PIns-P₆ with an apparent K_d of about 5μM (Fig. 13). The intracellular concentration of PIns-P₆ is estimated to be in the low micromolar range,
20 which is unlikely to interfere *in vivo* with the binding of tagmin to the polyphosphoinositides described herein. Presynaptic injection of higher concentrations of InsPP causes a reversible blockade of neurotransmitter release²¹. The present findings indicates that InsPP also acts by competing for the binding of tagmin to PIns-4,5-P₂ and/or PIns-3,4,5-P₃.

Binding of PIns-4,5-P₂ and PIns-3,4,5-P₃ to tagmin is highly specific, as shown by 1) lack of tagmin binding to liposomes containing PIns and PIns-4-P; 2)
30 saturable binding of PIns-4,5-P₂ to tagmin with 1:1 stoichiometry; 3) the competitive inhibition of the above binding reactions by the soluble inositol derivative InsP₆; and 4) the switch in specificity from PIns-3,4,5-P₃ to PIns-4,5-P₂ as a function of free calcium ion.

35 As the free calcium concentration is raised from

- 22 -

resting (basal) levels (\leq 30 nM) to about between 0.1 and 10 μ M, tagmin switches its specificity from PIns-3,4,5-P₃ to PIns-4,5-P₂. Many proteins of diverse function are known to contain C2 domains²⁹ or are known to bind InsPP.³¹ Therefore, they, too, have switchable lipid binding specificity, although the switch may be thrown by mechanisms other than calcium binding.

Polyphosphoinositide binding to tagmin and the calcium-dependent switch in its specificity is directly correlated to the mechanism of exocytosis. A PIns-specific transfer protein, a PIns-4-P 5-kinase (synthesizing PIns-4,5-P₂) and PInsP₂ or PInsP₃ are required for fusion to be triggered by calcium.^{26,36,37} Exocytosis is triggered when free calcium is above a threshold of about 20 μ M,³⁸ the same range at which tagmin binds PS and has switched from binding PIns-4,5-P₂ to PIns-3,4,5-P₃. Fig. 11. The need for PIns-specific transfer protein and PIns-4-P 5-kinase during priming^{26,37} is explained by the need to maintain a pool of PIns-4,5-P₂ to be available to bind tagmin when it releases PIns-3,4,5-P₃. This switch (and the binding to PS) is important in the rapid triggering of exocytosis that occurs after calcium levels rise. It is noted that the C2B domain of tagmin is known to bind β -SNAP and NSF in a Ca²⁺-independent fashion and assemble with them and α -SNAP and SNAREs into a putative docking and fusion particle for exocytosis.³⁵

Modulation of PIns-P₂ and tagmin binding is another mode of modifying synaptic transmission according to this invention, as PIns-P₂ is involved in secretion of synaptic vesicles. Binding of PIns-P₂ to tagmin also occurs to the carboxy-terminal portion as with β -SNAP and is inhibited by polyphosphoinositols (see Figure 8). Tagmin/ β -SNAP complex binds to PIns-P₂ with the same efficiency as the pure tagmin. Another aspect of this invention is therefore assays which detect changes in

- 23 -

- o PIns-P₂ binding to the cell free tagmin complexes of this invention and the method of decreasing synaptic transmission by decreasing or inhibiting PIns-P₂ binding to complexes comprising tagmin, β -SNAP or any of the other proteins which participate in docking to SNAREs.
- 5 Substances identified which decrease or inhibit PIns-P₂ and tagmin interactions may be useful to treat neurological or psychiatric diseases in which it is desirable to reduce synaptic transmission. Lithium, a well known drug for the treatment of bipolar manic-depressive disorders, acts by altering the inositol homeostasis and in particular by reducing the PIns-P₂ levels in the membrane. Babarain J.M., Proc. Natl. Acad. Sci., U.S.A., 91:5738-5739 (1994), Gain et al., Biochem. biophys. Acta., 1177:253-269 (1993). This invention
- 10 provides an assay for identifying other such substances which may be useful as adjuncts or in place of traditional therapies for bipolar depression which rely on lithium.
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The phosphatidylinositol pathway which causes phosphorylation at position 3 is significantly different from that resulting in phosphorylation at positions 4 and 5. Enzymes for the synthesis and degradation of these two different PIns-P₂ species are completely separated and non-overlapping in the cell. Accordingly, by targeting modulation of enzymes specific for one PIns-P₂ isomer over the other, this invention provides another means of specifically modulating synaptic transmission.

One embodiment of this invention relates to a method of treatment to improve synaptic transmission by modulating formation of tagmin-containing complexes, wherein calcium sensitivity of PIns-P₂/tagmin and PIns-P₃/tagmin complex formation is modulated by the addition of external substances.

Examples of "external substances" which may be used in this invention include several preferred categories of substances. A first preferred category of

- 24 -

external substances encompasses those that act by altering intracellular calcium concentrations, such as, for example, calcium channel blockers, including phenylalkylamines, dihydropyridines, benzothiazepines and diphenylpiperazines. Other calcium channel blockers are known in the art, such as those described by Goodman and Gilman's The Pharmacological Basic of Therapeutics 8th Eds. ((Eds. Goodman Gilman et al.) Pergamon Press 1990 p. 774-783). A second preferred category of external substances encompasses those that inhibit or enhance mobilization of calcium from intracellular calcium stores. Such substances include, for example, thapsigargin, inositol 1,4,5 trisphosphate ("IP₃") and 2,5-di(tert-butyl)-1,4-benzohydro quinone. Other such compounds are known in the art. A third preferred category of external substances useful in this method of the invention encompass substances which inhibit or enhance the synthesis of polyphosphoinositides and their soluble analogues including IP₆ and its derivatives, for example, lithium which reduces the level IP₃ and of polyphosphoinositides.

Assay Systems and Kits

The assay systems and kits of this invention comprise the reagents necessary to conduct the various binding assays described above which may be used to identify substances which reduce or inhibit complex formation. A typical assay kit of this invention would comprise β-SNAP or an amino acid sequence of β-SNAP capable of binding to tagmin, one or more containers each comprising an isoform of tagmin or an amino acid sequence of tagmin capable of binding β-SNAP, and optionally containers comprising separately one or more of NSF, α-SNAP, SNAREs complex, labelled specific antibodies, PI_nS-P₂, PI_nS-P, and one or more polyphosphoinositols to serve as controls to compare against test substances.

- 25 -

Components of the kits or assay systems may be provided in appropriate buffers, or may be lyophilized for later reconstitution and use. A preferred buffer is HEPES-KOH (20 mM), 100 mM KCl, dithiothreitol (0.1 mM) at pH 7.6. In particular the region of the amino acid sequence of tagmin extending from about residue 248 to about 421 or the entire cytoplasmic domain (amino acids 78-421) are particularly preferred for use in this invention because, as shown below, this region contains the binding site for β -SNAP and are stable in solution.

As discussed above assays are performed according to the invention by combining the complex components in an appropriate buffer, separating bound from free components and assaying for the presence of particular components in the complex. A preferred buffer for the binding reaction is HEPES-KOH (20mM, pH 7.6), potassium acetate (150 mM), glycerol (1%), octylglycoside (0.6 mM) and dithiothreitol (0.1 mM).

EXAMPLES

EXAMPLE 1. β -SNAP/Tagmin Complexes

In preliminary experiments, α -SNAP or β -SNAP were added to octylglucoside extracts of brain membranes, but only α -SNAP was recovered bound to the SNAREs. This was surprising, as either SNAP isoforms can bind when added to previously isolated SNAREs (Fig. 1c) and suggested that β -SNAP, but not α -SNAP, must bind with high affinity to an abundant membrane protein present in crude extracts but not in pure SNARE preparations. In fact, glutathione-S-transferase (GST)- β -SNAP fusion protein specifically binds a 65 kD polypeptide from crude extracts identified as synaptotagmin (tagmin) by Western blotting.

Confirming this, beads containing the cytoplasmic domain (Fig. 2a) of tagmin (residues 79 - 421

- 26 -

of tagmin I) linked to GST bind about 1 mole of β -SNAP/mole of tagmin (Fig. 2b, lane 3) at saturation, while neither α -SNAP (lane 2) nor the other ubiquitous SNAP isoform γ -SNAP⁹ bind. Only the carboxy-terminal portion of tagmin (residues 248-421; Fig 2a) containing the C2B domain binds β -SNAP (Fig. 2b, lane 9); the C2A domain-containing amino terminal portion (residues 96-265) that is known to bind calcium and acidic phospholipids¹⁰ does not bind β -SNAP (lane 8).

Tagmin/ β -SNAP/NSF complex is stable in the presence of $\text{Ca}^{2+}/\text{Mg}^{2+}$ ATP (right panel). GST fusion proteins of different tagmin constructs or GST alone were incubated with α -SNAP (lanes 2, 4, 7) or β -SNAP (lanes 3, 5, 7-13) with (lanes 4-7, 10-13) or without (lanes 1-3, 8, 9) addition of NSF. Three samples, identical to the one in lane 5, were further incubated in the presence of ATP γ S (lane 11), ATP (lane 12) or ATP + Ca^{2+} (lane 13). c) α -SNAP and β -SNAP binding to the SNARE complex. SNARE complexes were isolated from bovine brain detergent extract and incubated with increasing amounts of α -SNAP (closed squares) or β -SNAP (open squares).

METHODS: GST-fusion proteins containing the cytoplasmic domain of synaptotagmin I (aa. 79-421; 4 μg) or either the amino-terminal (GST-p65_{1,3}; aa. 96-265; 3 μg) or the carboxy-terminal (GST-p65_{3,5}; aa. 248-421; 3 μg) parts, were immobilized on GSH-agarose beads. Then 3 μg of His₆- α -SNAP³ or 3 μg of His₆- β -SNAP were diluted in 250 μl of 20 mM HEPES-KOH pH 7.6, 150 mM Acetate-K, 1% glycerol, 5 μM EDTA, 0.8% (w/v) octyl- β -D-glucopyranoside, 0.4 mg/ml ovalbumin, spun at 15,000 $\times g$ for 5 min and the supernatant was incubated with beads for 1 h at 4°C. Subsequently the beads were washed twice with 250 μl of the same buffer without ovalbumin. Some samples were further incubated for 1 h at 4°C with 5 μg of recombinant NSF³ in 250 μl of 20 mM HEPES-KOH pH 7.6, 150 mM Acetate-K, 1 mM MgCl₂, 5 μM EGTA, 0.5 mM ADP, 1% glycerol,

- 27 -

o 0.8% (w/v) octyl- β -D- glucopyranoside, 0.4 mg/ml ovalbumin. The beads were then washed in the same buffer without ovalbumin and samples analyzed in SDS-PAGE. The stability of the complex was tested by incubating the complex-bound beads for 30 min at 37°C in 20 mM HEPES-KOH pH 7.6, 150 mM Acetate-K, 1 mM MgCl₂, 1% glycerol, 0.5% (w/v) Triton X-100 (buffer A) containing 0.5 mM adenosine-5'-[γ -thio]triphosphate (ATP γ S)/1 mM EGTA, 0.5 mM ATP/1 mM EGTA, or 0.5 mM ATP/300 μ M free Ca²⁺.

For the determination of the α / β -SNAP binding to the SNARE complex, increasing amounts of SNAPs were diluted in 20 mM HEPES-KOH pH 7.6, 150 mM Acetate-K, 1% glycerol, 0.5% (w/v) Triton X-100 (buffer B), prespun and added to beads containing the SNARE complex isolated from bovine brain extract (250 μ g of protein/sample), by using a monoclonal anti-syntaxin antibody coupled to Protein G Fast Flow⁷. After 1 h at 4°C the beads were isolated, washed and the samples analyzed by Tris-urea/SDS-PAGE³. The amount of recovered proteins was evaluated by densitometric scanning of the Coomassie Blue R-250 stained bands. Data points (Figure 2) represent the average of three independent experiments.

Since NSF is known to bind to membranes in a SNAP-dependent manner,^{11,12} we tested whether NSF might bind to the β -SNAP-tagmin complex. NSF binds efficiently to the tagmin- β -SNAP complex (lane 5), while α -SNAP neither substitutes for β -SNAP (lane 4) nor interferes with this binding (lane 7).

NSF bound through α -SNAP to SNAREs hydrolyses ATP, releasing NSF and α -SNAP, with disruption of the SNARE complex⁷. However, complexes of NSF and β -SNAP with tagmin are stable in the presence of Mg²⁺-ATP, with (lane 13) or without (lane 12) calcium (0.3 mM), and these complexes form in the absence or presence (0.3 mM) of calcium (not shown). The lack of release of NSF with Mg²⁺-ATP suggests that β -SNAP and NSF are bound to tagmin

- 28 -

differently than in 20S particles,^{3,7} and implies that the tagmin/ β -SNAP/NSF "triple" complex should be stable *in vivo* under conditions in which ample ATP is present. Tagaya and colleagues¹³ have reported that NSF is present on synaptic vesicles, and is not released by Mg²⁺-ATP; the tagmin/ β -SNAP/NSF triple complex may explain their findings.

EXAMPLE 2. Evidence For Triple Complex
Involvement In Docking

METHODS: GST, GST-tagmin, GST-tagmin/ β -SNAP and GST-tagmin/ β -SNAP/NSF complexes, bound to GSH-agarose beads were prepared as described in Fig. 2. The complexes were resuspended in 20 mM HEPES-KOH pH 7.6, 150 mM Acetate-K, 1% glycerol, 0.8% (w/v) octyl- β -D-glucopyranoside, (Buffer C) containing 1 mM MgCl₂, 0.5 mM ADP, 1 mM phenylmethylsulfonylfluoride, 1 mM dithiothreitol and mixed in a final volume of 500 μ l with bovine brain extract (250 μ g of protein) prepared as described³ by using octyl- β -D-glucopyranoside as detergent. 10 μ g of His₆- α -SNAP or 10 μ g of His₆- β -SNAP were added as indicated. The samples were incubated for 1 h at 4°C, recovered by centrifugation at 5,000 \times g for 1 min and washed three times in buffer C containing 1 mM MgCl₂, 0.5 mM ADP. The pellets were analyzed by Tris-urea/SDS-PAGE, transferred to nitrocellulose and immunodecorated with a monoclonal antibody against syntaxin/HPC1 and with polyclonal antibodies against SNAP-25 and against rat VAMP⁷. Immunoreactive bands were visualized by using the Enhanced Chemoluminescence method (ECL, Amersham). Similar results were obtained by using Triton X-100 bovine membrane extract³ and by controlling the free calcium concentration to 1 μ M as described for calcium-dependent priming²³.

Consistent with this, assembled SNARE complexes

- 29 -

bind to the triple complex immobilized on beads, but only when α -SNAP was also added (Fig. 3, lane 8), underscoring the specificity of this interaction. Much less binding occurred in the absence of α -SNAP (lane 7). β -SNAP could substitute for α -SNAP but was not as potent (lane 9). In keeping with this, β -SNAP binds to SNAREs (Fig. 2c, open squares) with 2-3-fold lower affinity than does α -SNAP (closed squares); similar amounts of each (about 3 moles per mole of SNAP-25) bind at saturation. All three SNARE proteins (VAMP, syntaxin, and SNAP-25) bind together (lane 8), along with α -SNAP (not shown); the previously associated β -SNAP and NSF remain bound (not shown). Efficient binding of α -SNAP-SNARE complexes only occurred when NSF was present in the triple complex, and not to beads containing just GST-tagmin, or GST-tagmin- β -SNAP complexes (Fig. 3), and was not significantly affected by calcium.

Previously, it was reported⁷ that endogenous tagmin, isolated in low molar yield bound to the native synaptic SNARE complex, was displaced by excess α -SNAP, leading to the hypothesis that the SNARE complex could not simultaneously bind both tagmin and NSF/SNAPS. The recombinant tagmin cytoplasmic domain also binds weakly to the SNARE complex (Fig. 3, lane 1) and is also displaced by excess α -SNAP. Thus, the present data does not contradict but rather extend the previous findings with the important addition that it is the highly specific interaction of tagmin with β -SNAP that allows the SNARE complex to simultaneously bind tagmin, β -SNAP and NSF.

Because each of its subunits or close homologue is known to be required *in vivo* for exocytosis according to physiological^{4,18}, pathophysiological¹⁹ or genetic tests^{6,8,16}, this complex represents a specialized docking and fusion particle for regulated exocytosis at the synapse, and perhaps elsewhere.

- 30 -

EXAMPLE 3. Inhibition Of β -SNAP/Tagmin Complex Formation by Polyphospho-inositols.

Since it was found that the C2B domain also binds β -SNAP (Fig. 2b, lane 9), the effects of polyphospho-inositol compounds on the binding of β -SNAP to tagmin were tested (Fig. 4).

METHODS: 4 μ g of GST-tagmin immobilized on GSH-agarose beads were incubated for 15 min at 4°C with variable concentration of inositol 1,4,5-trisphosphate (InsP_3) or inositol 1,3,4,5-tetrakisphosphate (InsP_4) or inositol 1,3,4,5,6-pentakisphosphate (InsP_5) or inositol 1,2,3,4,5,6-hexakisphosphate (InsP_6) (Sigma). Then β -SNAP (4 μ g) diluted in buffer C containing 0.4 mg/ml ovalbumin and previously spun at 15,000 \times g for 5 min was added. The samples (250 μ l final volume) were incubated for 1 h at 4°C, washed twice with 250 μ l of buffer C and then analyzed by SDS-PAGE. The β -SNAP bound to GST-tagmin was determined by scanning of the Coomassie Blue R250 stained gel as described before (see legend Fig. 2).

These same compounds blocked β -SNAP binding with the same rank order and with similar potency to that reported for their binding to tagmin²⁰. Preformed tagmin- β -SNAP and tagmin- β -SNAP-NSF triple complexes are stable in the presence of these substances. This implies that active polyphosphoinositols introduced *in vivo* would only block the assembly of new triple complexes and new docking and fusion particles formed from them. Consistent with this, polyphosphoinositols potently blocks transmission when injected presynaptically, following a lag time of 15-45 min²¹. While this can be explained by the effects of these compounds on the binding of β -SNAP to tagmin, additional effects in synapses cannot be excluded.

- 31 -

° EXAMPLE 4. Mg²⁺ ATP Dependent Disassembly Of the
Specialized Docking And Fusion Particle

5 NSF entering the possible docking and fusion particle bound in the "triple" complex with β -SNAP and tagmin can hydrolyze ATP to release VAMP as shown by cleavage by tetanus toxin²² (Fig. 5, open squares). ATP γ S does not substitute for ATP (open triangles), and an ATP-hydrolysis deficient mutant of NSF (E329Q/D604Q double 10 mutant)¹¹ will substitute for NSF in assembling the particle, but produce particles resistant to Mg²⁺ATP. These results imply that this new docking and fusion particle is not a dead-end complex, i.e., a particle containing a non-functional NSF molecule unable to disrupt the SNARE complex.

15 METHODS: The complex of GST-tagmin/ β -SNAP/NSF with SNAREs and α -SNAP was assembled from Triton X-100 bovine brain membrane extract³ as described in Fig. 1 and 2 and washed twice with buffer B, containing 1 mM MgCl₂, and 0.5 mM ADP. The particle-containing beads (15 μ l per sample) were then added to 235 μ l of pre-warmed buffer A 20 supplemented with 400 nM of the recombinant catalytic subunit of tetanus toxin²² and containing alternatively 0.5 mM ATP γ S/1 mM EGTA, 0.5 mM ATP/1 mM EGTA, or 0.5 mM ATP/300 μ M free Ca²⁺ (obtained by buffering with 1 mM EGTA). At the indicated times samples were chilled on ice 25 and centrifuged at 5,000 \times g for 1 min. Pellets were analyzed by Tris-urea/SDS-PAGE, transferred to nitrocellulose and immunodecorated with a polyclonal antibody against rat VAMP⁷. Immunoreactive bands were visualized by ECL and quantified by scanning of the 30 resulting X-ray film. VAMP content at different incubation times was expressed as percentage of the amount present at time zero. Analysis of supernatants showed only trace amounts of VAMP independent of the time of incubation, as expected.

35 Calcium does not affect the rate of ATP-

- 32 -

dependent disassembly of the particle (Fig. 5, closed squares). Studies of calcium-triggered exocytosis in patch-clamped and in permeabilized neuroendocrine cells have revealed two distinct stages in this process²³⁻²⁵. A slow ATP-dependent, but calcium-independent process termed "vesicle priming", is followed by a rapid ATP-independent step triggered by calcium.

Our observations favor the possibility that priming includes the NSF-ATPase-dependent disruption of the proposed synaptic docking and fusion particle, producing a latent fusogenically-active state awaiting calcium. If this assignment is correct, then, like priming, disruption of the docking and fusion particle should be independent of calcium, as we observed (Fig. 5). This view in no way contradicts the additional need for ATP during the priming stage to maintain a pool of phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-P₂) for later fusion after calcium activation²⁶. The protein components that might constitute the latest fusogenically-active state that be produced when ATP is hydrolyzed by NSF are unknown but could be satisfied by tagmin which possesses a calcium binding site and an affinity for β -SNAP.

EXAMPLE 5. Binding Of Liposomes to Complexes

Methods: Liposomes (175 μ g/ml) were made by pure 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (PC) or PC and 1,2-dioleoy-sn-glycero-3-[phospho-L-serine] (PS) (3:1 w/w) or PC containing 1% w/w 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-D-myo-inositol (PI) or 1% (w/w) 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-D-myo-inositol-4-phosphate (PIns-P) or 1% (w/w) 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-D-myo-inositol-4,5-bisphosphate (PIns 4,5-P₂). Liposomes contained 1 μ Ci/ml of 1,2-dipalmitoyl-sn-glycero-3-phospho-[N-methyl-3H]-

- 33 -

choline (66 Ci/mmol, Amersham) as tracer. Lipids were purchased from Avanti Polar Lipids (PC, PS and PI) or Boehringer Mannheim GmbH (PIns-P and PIns-P₂). Phospholipids were dried by a gentle flow of argon on a 4 ml glass vial and then the residual traces of solvents were eliminated under vacuum for 15 minutes at room temperature. The lipid film were resuspended in 20 mM HEPES-KOH pH 7.6, 100 mM KC1 mM DTT (buffer A) by vigorous stirring for 10 minutes and the top of the solution were layered with argon. Liposomes were prepared either by sonication in a Branson cup sonicator (intensity setting 7, duty cycle 50%, 8 minutes)²⁸ followed by liposomes purification on Sephadex G-25 M (Pharmacia) or by freeze-thawing (5 times) and extrusion (19 times) through a 100 nm pore carbonate membrane (Avestin, Ottawa, Canada)⁴². Large aggregates were eliminated by centrifugation at 15,000g for 10 minutes. The liposomes are stored at 4°C and are used within one week. The FPLC-purified cytoplasmic domain of synaptotagmin 1 (tagmin, aa. 79-421; 15 µg) or its fragments, correspondent to the amino-terminal (C2A; aa. 96-265; 11 µg) and the carboxy-terminal (C2B; aa. 248-421; 11 µg) portions, fused with glutathione-S-transferase (GST) or GST alone (8 µg) were immobilized on 20 µl 50% GSH-agarose beads and washed twice with 500 µl of buffer A containing alternatively 2 mM EGTA or 100 µM free calcium buffered with 2 mM EGTA. Subsequently 100 µl of [³H]-labelled liposomes containing the desired concentration of free calcium were added and the beads were incubated for 20 minutes at room temperature with vigorous shaking. In selected samples, liposome binding were performed in the presence of 5 µM inositol 1,2,3,4,5,6-hexakisphosphate (InsP₆, Sigma). Samples were centrifuged at 3000g for 2 minutes and the pellets were washed three times with buffer A plus or minus 100 µM free calcium. The bound liposomes were solubilized with 0.4 ml of 10% SDS and radioactivity

- 34 -

quantified by scintillation counting. The experimental findings were confirmed in at least three independent experiments. The molecular identity of the inositol phospholipids were confirmed by mass spectrometry and by thin-layer chromatography-immunostaining assay (Higashi et al., J. Biochem. (Tokyo) 95, 1517 1984) with an anti-PIn₆P₂ specific antibody (Matuoka et al., Science 239:640-643 (1988)).

As shown in Fig. 6, only liposomes containing PIns-P₂ bound significantly to tagmin, other inositol containing phospholipids (PI and PIns-P) show only minor binding activity. Evidence that PIns-P₂ binds to the same region of tagmin as does β -SNAP is provided by the inhibition by inositol 1,2,3,4,5,6-hexakisphosphate (IP₆) of PIns-P₂ binding to tagmin (Fig. 8B). The lack of any inhibition of PS binding by IP₆ further confirms the different specificity of binding. The PIns-P₂ binding site is localized to the carboxy-terminal domain (C2B) and accounts for the entire PIns-P₂ binding of synaptotagmin. In fact, the amino terminal domain, C2A, shows barely detectable binding activity which is not distinguishable from the fusion protein alone (GST) (Fig. 7, lower panel). The functionality of the C2A domain is demonstrated from the calcium-dependent binding of this region of synaptotagmin (Fig. 7, upper panel).

EXAMPLE 6. Specific Calcium-dependent Binding of Synaptotagmin to PInsPs-Containing Liposomes.

To test if the InsPP binding site on tagmin can also bind corresponding lipid phosphoinositides, beads containing glutathione S-transferase (GST) linked to tagmin I (residues 79-421) were incubated with liposomes containing phosphatidylcholine (PC) and different inositol phospholipids (1 % (w/w)) at close to the physiological concentration of free Mg²⁺ (0.5-1 mM).

- 35 -

METHODS: Liposomes (175 µg of lipid/ml) were made from pure 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (PC) or PC and in addition 1% (w/w) of 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-D-myo-inositol (PIns) or 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-D-myo-inositol-4-phosphate (PIns-4-P) or 1-stearoyl-2-arachidonoyl-sn-glycero-3-phospho-D-myo-inositol-4,5-bisphosphate (PIns-4,5-P₂) or 1,2 dipalmitoyl sn-glycero-3-phospho-D-myo-inositol -3,4-bisphosphate (PIns-3,4-P₂ or 1,2 dipalmitoyl sn-glycero-3-phospho-D-myo-inositol -3,4,5-trisphosphate (PIns-3,4,5-P₃) and 1 µCi/ml of 1,2-dipalmitoyl-sn-glycero-3-phospho-[N-methyl-³H]-choline (66 Ci/mmol, Amersham) was added as a tracer. Lipids were purchased from Avanti Polar Lipids (PC and PIns) or Boehringer Mannheim GmbH (PIns-4-P and PIns-4,5-P₂) or Matreya Inc (PIns-3,4-P₂). PIns-3,4,5-P₃ was synthesized as described before⁴¹ and its identity was confirmed by ¹H- and ³¹P-NMR. Lipids were dried by a gentle flow of argon, dissolved in 200 µl of 100 % ethanol and then kept under vacuum for 30 min. at room temperature. The homogeneous lipid film was then resuspended in 20 mM HEPES-KOH pH 7.6, 100 mM KCl, 0.2 mM dithiothreitol (DTT) (buffer A) by vigorous stirring for 10 minutes, after overlaying the top of the solution with argon. Liposomes were prepared either by sonication²⁸ followed by liposome purification on Sephadex G-25 M (Pharmacia) or by extrusion⁴². Large aggregates were eliminated by centrifugation at 15,000g for 10 minutes. Purified GST-fusion proteins containing the cytoplasmic domain of tagmin 1 (aa. 79-421; 15 µg) or GST alone (8 µg) were immobilized on 20 µl 50% glutathione-agarose beads (Sigma). The beads were then washed twice with 500 µl of the same buffer containing 1.0 mM free Mg²⁺ and either 2 mM EGTA or 100 µM free calcium buffered with 2 mM EGTA. Subsequently, 100 µl of [³H]-labeled liposomes were added and the beads were incubated for 30 minutes at room

- 36 -

temperature with vigorous shaking. Beads were centrifuged at 3000g for 2 minutes and washed three times with buffer A containing 1.0 mM free Mg²⁺ plus or minus 100 μM free calcium. The bound liposomes were solubilized with 0.3 ml of 10% SDS and radioactivity quantified by scintillation counting. Data shown are the average of three independent experiments ± s.d.

Figure 10A shows the binding specificity of GST-tagmin to liposomes. GST-tagmin was incubated with liposomes containing PC or PC together with distinct phosphoinositides (as indicated) in the presence or absence of calcium ions (shaded bars, 2 mM EGTA; filled bars, 100 μM free Ca²⁺). Lipid binding was quantified by liquid scintillation counting of the radioactive PC used as tracer and expressed as % of total radioactivity used. Specific binding was calculated by subtracting the non-specific lipid interaction of GST ($2.4 \pm 0.7\%$) from individual samples. Similar results were obtained both with small and large unilamellar vesicles, thus suggesting that size and curvature of the liposome do not influence the binding.

EXAMPLE 7. Calcium dependency of PIns-4,5-P₂ and PIns-3,4,5-P₃ binding to tagmin.

PIns-4,5-P₂ and PIns-3,4,5-P₃ were incorporated into separate liposome populations, labeled either with a [¹⁴C] or with a [³H]-PC tracer. Binding of each vesicle type to tagmin-containing beads was determined in a mixed incubation as a function of free calcium concentration (Fig. 11). GST-tagmin beads were simultaneously incubated with two populations of PC liposomes either containing PIns-4,5-P₂ or PIns-3,4,5-P₃ at variable Ca²⁺ concentrations (See Figure 11, open circles, PIns-4,5-P₂; closed squares, PIns-3,4,5-P₃).

METHODS: PC liposomes (350 μg of lipid/ml) containing either 1% (w/w) of PIns-4,5-P₂ and 0.1 μCi/ml

- 37 -

o of 1-palmitoyl-2-[1-¹⁴C]palmitoyl-sn-glycero-3-phosphocholine (55 mCi/mmol, Amersham) or 1% (w/w) of PIns-3,4,5-P₃ and 1 μCi/ml of 1,2-dipalmitoyl-sn-glycero-3-phospho-[N-methyl-³H]-choline (66 Ci/mmol, Amersham) were prepared as above. The two liposome populations were pre-mixed at a 5 1:1 ratio (final lipid concentration 175 μg/ml) and free Ca²⁺ concentrations ranging from 1 nM to 300 μM in the presence of 0.5 mM free Mg²⁺ were obtained by adding the suitable amount of calcium or magnesium chloride to 2 mM EGTA in buffer A. GST-tagmin beads were then incubated 10 with the mixed liposomes for 30 min. at room temperature and washed in buffer A containing the corresponding amount of free Ca²⁺. [³H]/[¹⁴C] radioactivity associated with the pellet was then determined by scintillation counting.

Tagmin loses the capacity to bind PIns-3,4,5-P₃ between about 0.1 and 1 μM free calcium. It gains the capacity to bind PIns-4,5-P₂ progressively above about 1 μM free calcium with maximum binding reached only in the 100 μM range. The calcium dependency of the switch of specificity was not significantly affected by altering the 20 concentration of PIns-4,5-P₂ or PIns-3,4,5-P₃ in the liposome. However, magnesium (0.5-1.0 mM) was required for the complete switch. In the absence of Mg²⁺, the calcium dependency of the binding of tagmin to PIns-4,5-P₂-containing liposomes was less pronounced, as was the 25 calcium dependent reduction of the interaction of PIns-3,4,5-P₃ with tagmin.

EXAMPLE 8. Tagmin domains and Polyphosphoinositides binding specificity.

30 Beads containing bacterially expressed GST-tagmin, its GST-C2A or its GST-C2B domains were incubated with liposomes containing 1 % (w/w) PIns-4,5-P₂ or PIns-3,4,5-P₃ (Fig. 12).

35 The PInsPs binding site on tagmin is localized

- 38 -

o to the C2B domain. GST fusion proteins containing tagmin or its amino terminal (C2A; aa. 96-265; 10 µg) or the carboxy-terminal (C2B; aa. 248-421; 10 µg) domains were incubated with liposomes containing PC and either 25% (w/w) 1,2-dioleoy-sn-glycero-3-phospho-L-serine (PS) (Figure 12, panel A) or 1% (w/w) PI_ns-4,5-P₂ (Figure 12, panel B) or 1% (w/w) PI_ns-3,4,5-P₃ (Figure 12, panel C) (shaded bars, 2 mM EGTA; filled bars, 100 µM free Ca²⁺). Liposome binding to tagmin and its domains was determined as in Fig. 10A.

The PI_ns-4,5-P₂ and the PI_ns-3,4,5-P₃-directed binding of liposomes occur with the C2B domain of tagmin, with an efficiency similar or higher to the full length tagmin cytoplasmic domain (Fig. 12B and C).

EXAMPLE 9. Binding Kinetics of Micellar PI_ns-4,5-P₂ to Recombinant and Native Tagmin.

The binding of individual molecules of PI_ns-4,5-P₂ (as distinct from liposomes containing PI_ns-4,5-P₂) to tagmin could be measured directly because of the availability of radiolabeled PI_ns-4,5-P₂.

GST-tagmin (Fig. 13, filled circles) or GST alone (Fig. 13, filled triangles) were incubated at increasing concentration of radioactive PI_ns-4,5-P₂ in detergent micelles in the presence of 2 mM EGTA, as shown in Figure 13A. Samples were analyzed as described in Example 6. Panel A of Fig. 13 shows the tagmin/PI_ns-4,5-P₂ molar ratio as function of the micellar PI_ns-4,5-P₂ concentration. Dashed line (open circles) represents the PI_ns-4,5-P₂ specifically associated with tagmin. Parallel experiments using Triton X-100 (0.02% w/v) gave the same results.

GST-tagmin was incubated in the presence of saturable amount of PI_ns-4,5-P₂ (6 µM) with increasing amount of InSP₆ in the presence of 2 mM EGTA, as shown in

- 39 -

Figure 13B.

Immunoprecipitated native tagmin (Fig.13C, filled circles) or anti-tagmin antibody alone (Fig.13C, filled triangles) were incubated with radioactive PIns-4,5-P₂ in detergent micelles in the presence of 2 mM EGTA, 5 as shown in panel C. Dashed line (open circles) represents the PIns-4,5-P₂ specifically associated with tagmin.

SDS-PAGE profile of the immunopurified native tagmin used in panel C of Figure 13. In addition to the tagmin monomer with an apparent molecular weight (Mr) of 65 kDa, a SDS-resistant tagmin dimer is also visible⁴⁰.

METHODS: For the determination of the apparent affinity constant and the total number of binding sites for PIns-4,5-P₂ on tagmin, 1,2-dioleoy-sn-glycero-3-phospho-D-myo-inositol-4,5-bisphosphate [myo-inositol-2-³H(N)] (7 Ci/mMole; NEN) and unlabelled PIns-4,5-P₂ were mixed and dried with a gentle flow of argon. The lipid film was then dissolved in 200 µl of 100 % ethanol and the trace amounts of solvent were eliminated under vacuum. PIns-4,5-P₂ was solubilized at a final concentration of 20 µM (20 Ci/Mole) in buffer A containing 0.8 % (w/v) OG in the presence of 2 mM EGTA and sonicated as above. For the experiments shown in Fig. 13, panels C) and D) native tagmin (5 µg/sample) was immunoprecipitated from octyl-β-D-glucopyranoside (OG) extract of bovine brain cortex³⁵ with an anti-tagmin monoclonal antibody (M48)³³ covalently coupled to protein G-Sepharose Fast Flow (Pharmacia). After overnight incubation at 4°C, the beads were washed extensively with buffer A containing 0.5 M NaCl, 0.8 % OG and then rinsed in buffer A containing 0.8 % OG. Monomeric PIns-4,5-P₂ in detergent micelles were mixed with immunopurified native tagmin or immobilized GST-tagmin (6 µg) or control beads, incubated 30 min. at 4°C 30 in the presence of 2 mM EGTA and then analyzed as 35

- 40 -

described in Example 6.

For Fig. 13D, beads were analyzed by SDS-PAGE and the proteins were stained with Coomassie Blue R. Competition experiments were carried out by incubating GST-tagmin-containing beads with saturable amount of [³H]-PIns-4,5-P₂ (6 μM) premixed with increasing concentrations of InsP₆ (1 nM-1 mM) in buffer A containing 0.8 % OG, 2 mM EGTA for 30 minutes at 4°C. After washing with the same buffer, the amount of radioactivity was determined and results expressed as % of the binding in absence of InsP₆.

Radiolabeled PIns-4,5-P₂ was dispersed in octyl-β-D-glucopyranoside (OG) micelles using a vast excess of detergent to ensure that there would be at most one molecule of PIns-4,5-P₂ per detergent micelle. At saturation, almost exactly one mole of PIns-4,5-P₂ was bound per mole of tagmin (Fig. 13A), with an apparent Kd of about 1 μM. Unlike the binding of tagmin to PIns-4,5-P₂ contained in lipid bilayer vesicles, the binding of micellar PIns-4,5-P₂ monomers was independent of calcium. InsP₆ competes with this binding, with a Ki of about 10 μM. Fig. 13B. Full length native tagmin, immunopurified from brain cortex with an antibody directed against its first C2 homology domain³³ (Fig. 13D) shows the saturable and stoichiometric binding properties for PIns-4,5-P₂ (Fig. 13C) as was observed with the recombinant protein. InsP₆ inhibits the interaction of micellar PIns-4,5-P₂ monomers with the native protein, but less efficiently than observed with the recombinant tagmin (Ki ≥ 100 μM).

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- 41 -

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10 All of the foregoing references cited herein are incorporated herein by reference in their entirety.

While we have hereinbefore described a number of embodiments of this invention, it is apparent that the basic constructions can be altered to provide other 15 embodiments which utilize the methods and devices of this invention. Therefore, it will be appreciated that the scope of this invention is defined by the claims appended hereto rather than by the specific embodiments which have been presented hereinbefore by way of example.

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- 44 -

◦ WE CLAIM:

5 1. A cell free protein complex comprising an amino acid sequence of tagmin bound to at least one other molecule selected from the group consisting of PIns-P₂, PIns P₃ and an amino acid sequence of β-SNAP which binds tagmin.

10 2. The complex according to claim 1 comprising a tagmin binding amino acid sequence of β-SNAP and a β-SNAP binding sequence of tagmin.

15 3. The complex according to claim 2 further comprising at least one other protein selected from the group consisting of NSF, α-SNAP, syntaxin, VAMP, SNAP25, PIns-P₂ and PIns-P₃.

20 4. The complex according to claim 2 wherein the amino acid sequence of beta-SNAP is bound to the carboxy terminal portion of the tagmin amino acid sequence.

25 5. The complex according to claim 4 wherein the carboxy terminal portion of tagmin bound to beta-SNAP comprises an amino acid sequence selected from the group consisting of amino acid residues about 79-421 of tagmin I, amino acid residues about 248-421 of tagmin I, amino acid residues about 249 to 422 of tagmin II; amino acid residues about 405-588 of tagmin III; amino acid residues about 263-425 of tagmin IV; amino acid residues about 116 to 279 of tagmin V; amino acid residues about 338-511 of tagmin VI; amino acid residues about 242 to 403 of tagmin VII; and amino acid residues 177 to 255 of tagmin VIII.

- 45 -

6. The complex according to claim 5 wherein the carboxy terminal portion of tagmin I bound to beta-SNAP comprises amino acid residues about 248 to 421.

5 7. The complex according to claim 1 wherein the amino acid sequence of the tagmin comprises the full length sequence of the tagmin.

10 8. The complex according to claim 1 wherein the amino acid sequence of β -SNAP comprises the full length sequence of β -SNAP.

15 9. The complex according to claim 8 wherein the amino acid sequence of β -SNAP comprises amino acid 25 to 45 of β -SNAP.

10 10. The complex according to claim 5 wherein the tagmin amino acid sequence is provided as a recombinantly expressed fusion protein.

20 11. The complex according to claim 5 further comprising NSF.

25 12. The complex according to claim 1 wherein the tagmin amino acid sequence is bound to a solid support.

13. The complex according to claim 3 comprising β -SNAP, tagmin and NSF.

30 14. The complex according to claim 3 comprising β -SNAP, tagmin, NSF and α -SNAP.

35 15. The complex according to claim 1 comprising β -SNAP, tagmin, NSF, α -SNAP and at least one SNARE protein.

- 46 -

16. The complex according to claim 1 comprising tagmin and PIns-P₂, or tagmin and PIns-P₃.

17. A kit for identifying compounds capable of inhibiting synaptic transmission, said kit comprising a first container comprising an amino acid sequence of beta-SNAP capable of binding to a tagmin and at least one container comprising a beta-SNAP binding protein comprising an amino acid sequence present in at least one isoform of tagmin which amino acid sequence binds beta-SNAP, and wherein the beta-SNAP and beta-SNAP binding protein are present in amounts sufficient to assess the formation of beta-SNAP and tagmin complexes in the presence of a test compound.

18. The kit according to claim 17 comprising a plurality of containers comprising beta-SNAP binding protein, each container comprising an amino acid sequence of a different isoform of tagmin.

19. The kit according to claim 17 further comprising additional proteins for assessment of complexing formation, said proteins being selected from the group consisting of NSF, α -SNAP and SNARE proteins in amounts sufficient to form complexes with the beta-SNAP and the amino acid sequence of tagmin.

20. The kit according to any one of claims 17, 18 or 19 wherein the beta-SNAP binding amino acid sequence is bound to a solid support.

21. The kit according to claim 17 wherein the amino acid sequence of tagmin isoforms is a fragment of tagmin consisting essentially of the carboxy terminal beta-SNAP binding segment.

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- 47 -

22. The kit according to claim 21 wherein the amino acid sequence of tagmin consists essentially of amino acid residues about 248 to about 421 of tagmin I.

5 23. The kit according to claim 17 wherein the amino acid sequence of tagmin is present as a fusion protein.

10 24. A method of identifying substances capable of inhibiting synaptic transmission, said method comprising:

15 (a) combining the components to form any of the complexes selected from the group consisting of β -SNAP/tagmin; β -SNAP/tagmin/NSF; β -SNAP/tagmin/NSF/ α -SNAP; β -SNAP/tagmin/NSF/ α -SNAP/SNARE protein; PIns-P₂/tagmin; and PIns-P₃/tagmin with a test substance under conditions which allow for complex formation; and

(b) detecting the formation of complexes.

20 25. The method according to claim 24 wherein a plurality of different isoforms of tagmin are tested for complex formation with beta-SNAP in the presence of the test substance.

25 26. The method according to claim 24 wherein the beta-SNAP binding protein is bound to a solid support.

30 27. The method according to claim 25 wherein the beta-SNAP binding protein is a fusion protein consisting essentially of the carboxy terminal portion of tagmin and an additional amino acid sequence.

35 28. The method according to claim 27 wherein the carboxy terminal portion of tagmin consists essentially of amino acid residues about 248 to about 421 of tagmin I.

- 48 -

29. The method according to claim 28 wherein additional amino acid sequence bound to the carboxy-terminal sequence of tagmin is a GST sequence.

5 30. A method of modulating synaptic transmission comprising exposing a neuronal cell to a substance which inhibits binding of beta-SNAP to tagmin.

10 31. A method of modulating synaptic transmission comprising exposing a neuronal cell to a substance which inhibits binding of PIns-P₂ to tagmin.

32. The method of claim 31 wherein the substance inhibits binding of PIns-P₁ to tagmin.

15 33. A method of modulating synaptic transmission comprising exposing a neuronal cell to a substance which inhibits formation of a complex selected from the group consisting of β-SNAP/tagmin; β-SNAP/tagmin/NSF; β-SNAP/tagmin/NSF/α-SNAP; and β-SNAP/tagmin/NSF/α-SNAP/SNARE protein; PIns-P₂/tagmin; and PIns-P₃/tagmin.

20 25 34. A method of treating a neurological or psychiatric disorder related to improper synaptic transmission by modulating formation of tagmin complex s.

30 35. The method according to claim 34 wherein the tagmin complexes are selected from the group consisting of β-SNAP/tagmin; β-SNAP/tagmin/NSF; β-SNAP/tagmin/NSF/α-SNAP; and β-SNAP/tagmin/NSF/α-SNAP/SNARE protein; PIns-P₂/tagmin; and PIns-P₃/tagmin.

35 36. The method according to claim 34 wherein modulation of tagmin complex formation is affected by altering calcium levels by means of altering permeability

- 49 -

° of a calcium channel.

37. The method according to claim 34 wherein
calcium sensitivity of PI_ns-P₂/tagmin and PI_ns-P₃/tagmin
complex formation is modulated by addition of external
5 substances.

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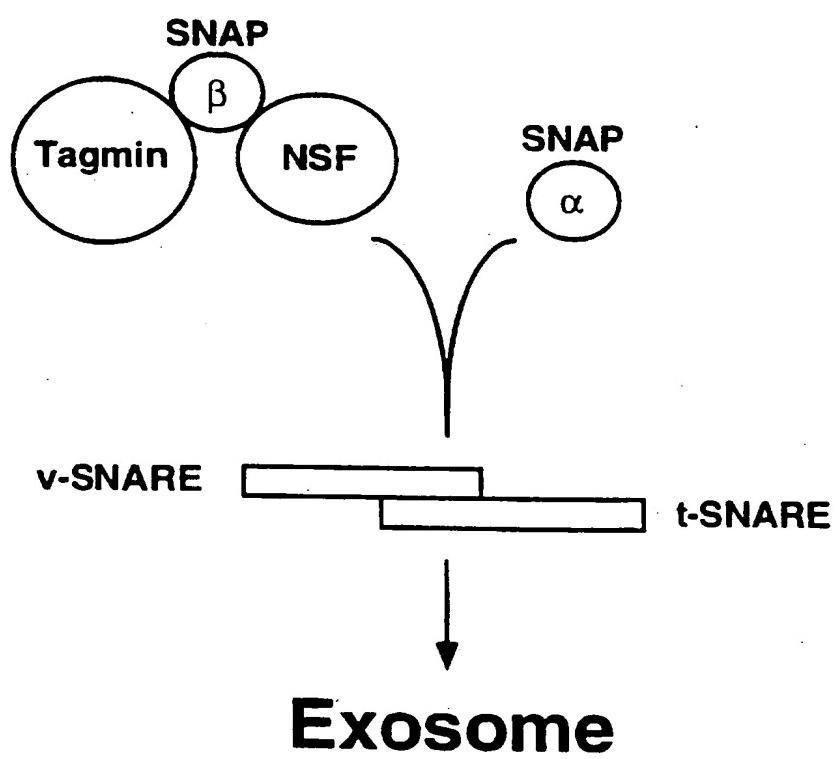
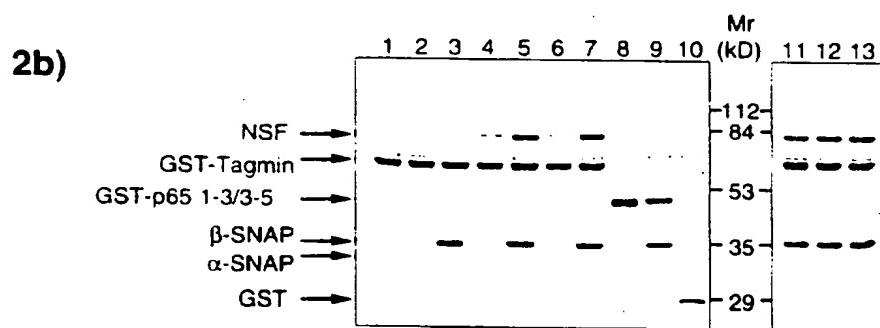
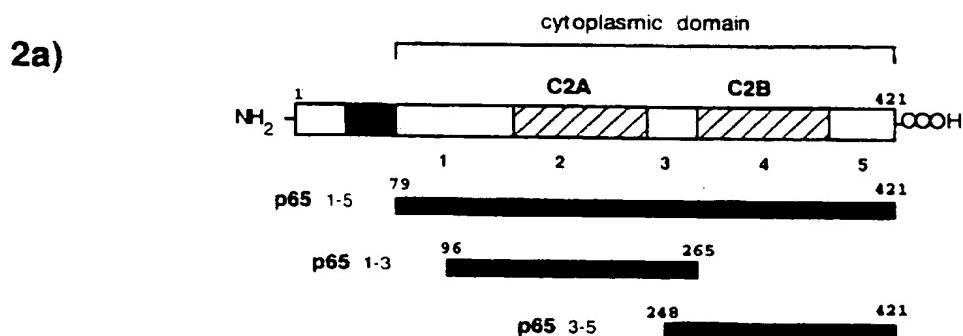


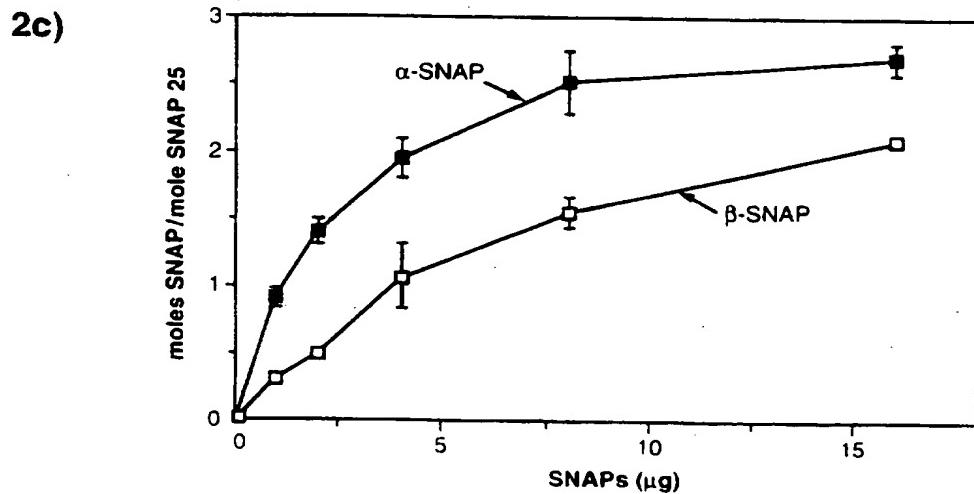
FIG. 1

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2/13



GST-Tagmin	+	+	+	+	+	+	+	-	-	-	+	+	+	GST-Tagmin
GST-p65 1-3	-	-	-	-	-	-	-	+	-	-	+	+	+	β-SNAP
GST-p65 3-5	-	-	-	-	-	-	-	-	+	-	+	+	+	NSF
GST	-	-	-	-	-	-	-	-	-	+	+	+	+	Mg ⁺⁺
α-SNAP	-	+	-	+	-	+	-	-	-	-	+	-	-	ATP γ S
β-SNAP	-	-	+	-	+	-	+	+	+	+	-	+	+	ATP
NSF	-	-	-	+	+	+	+	-	-	+	-	-	+	Ca ⁺⁺

**FIG. 2**

3 / 13

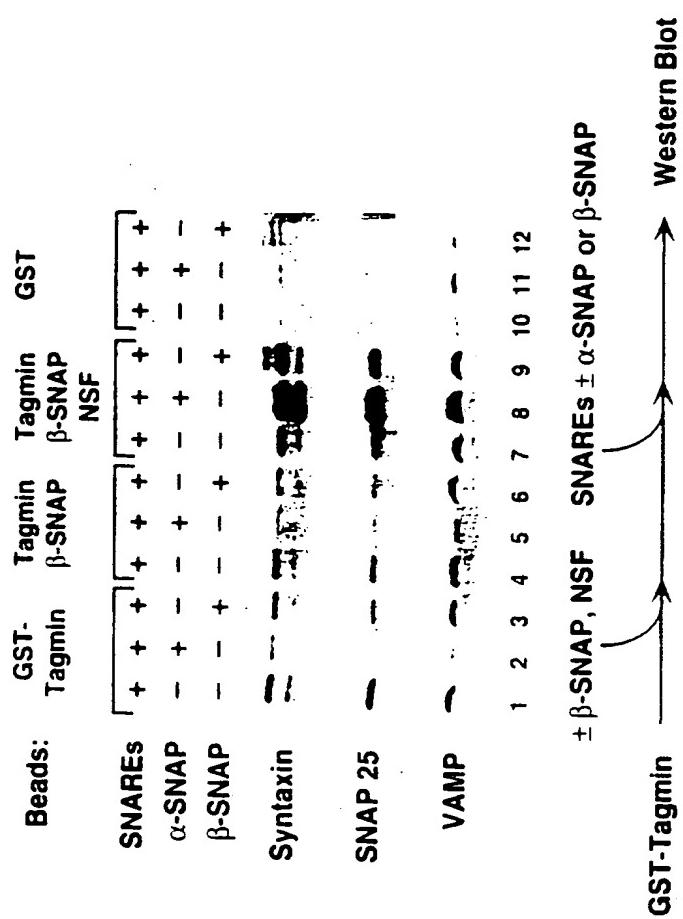


FIG. 3

4/13

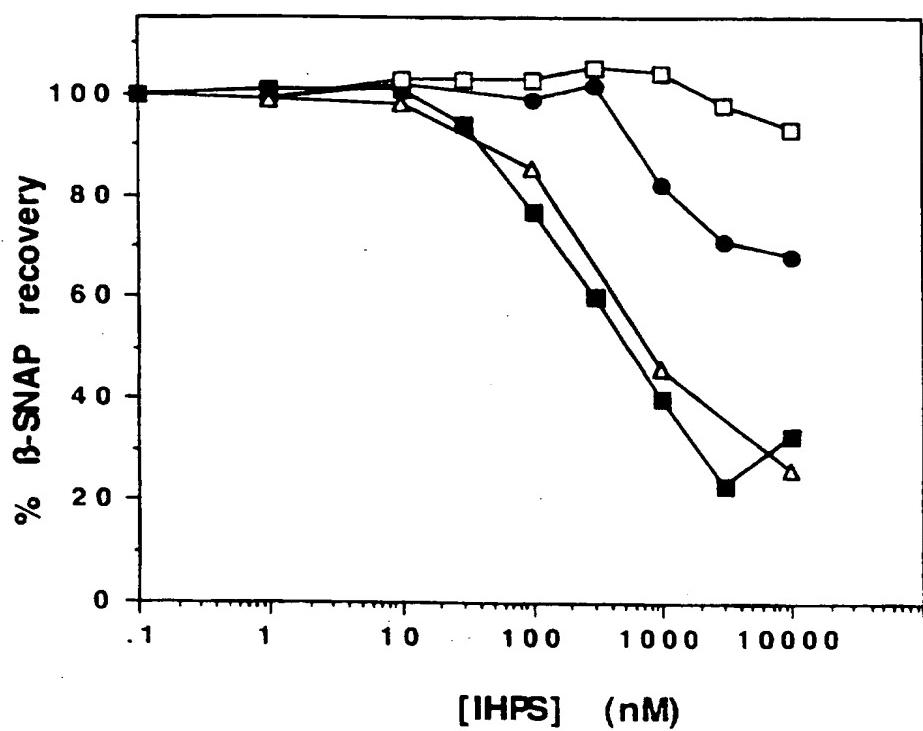


FIG. 4

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5/13

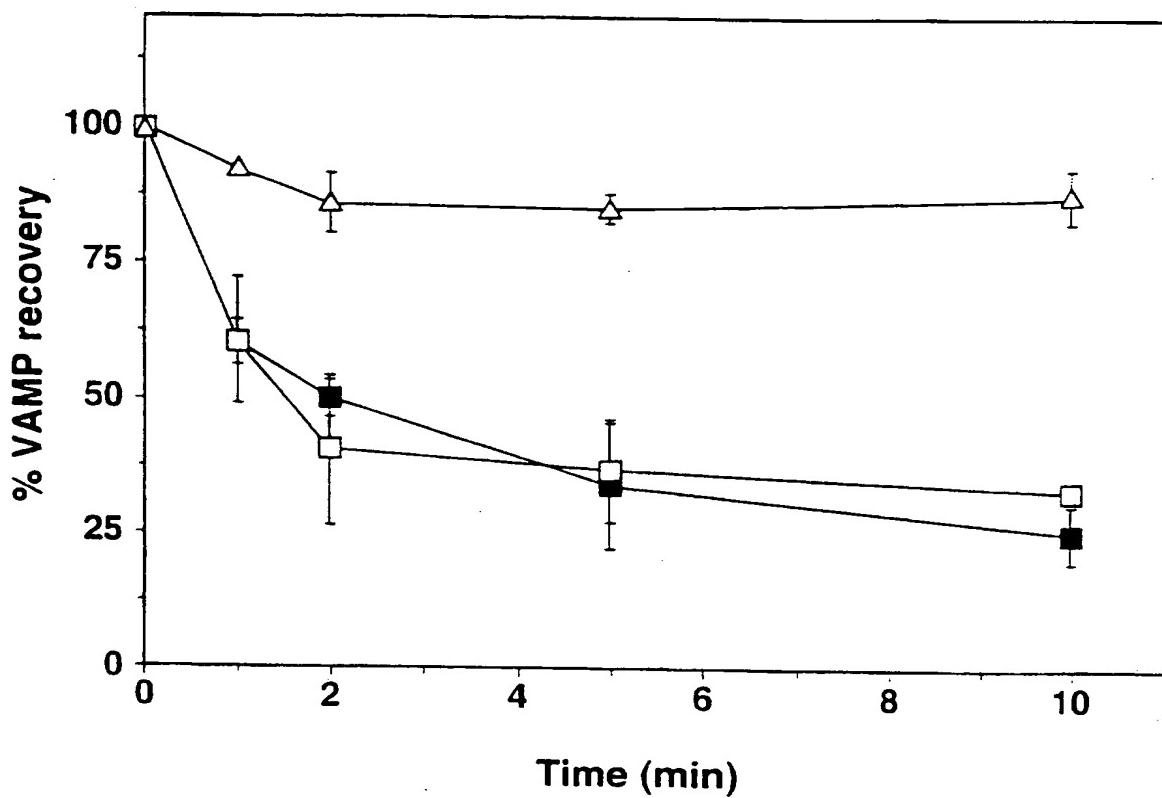


FIG. 5

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6/13

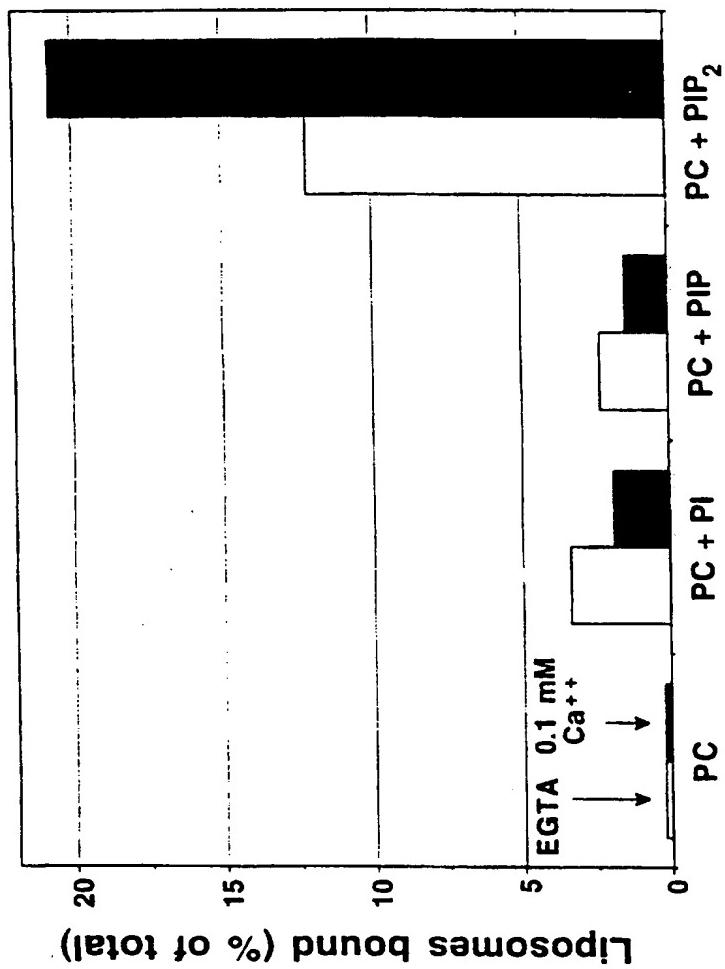


FIG. 6

7/13

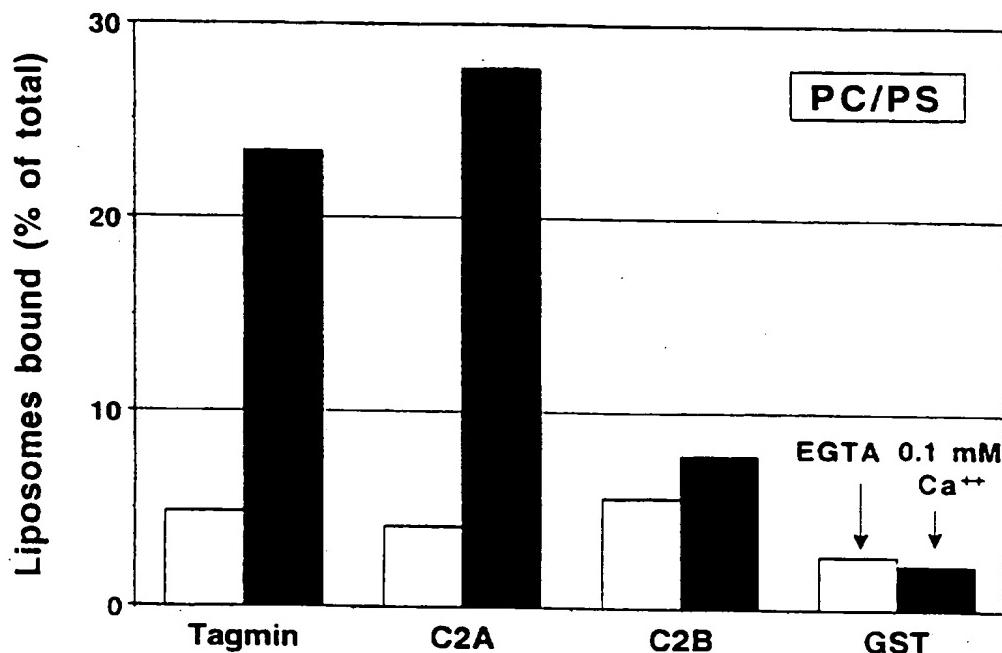


FIG. 7A

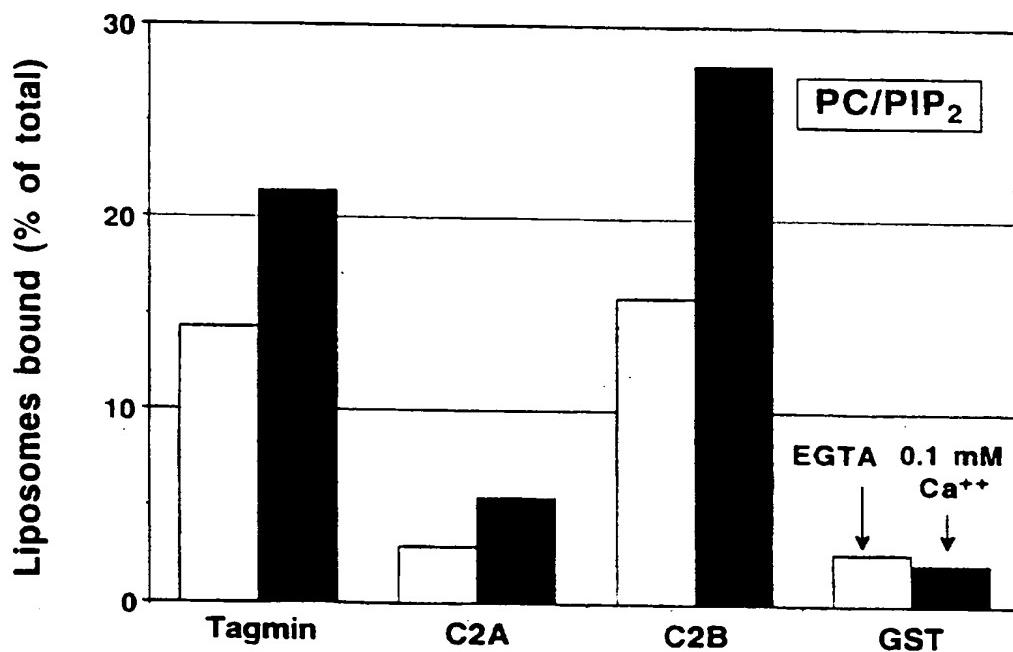


FIG. 7B

8/13

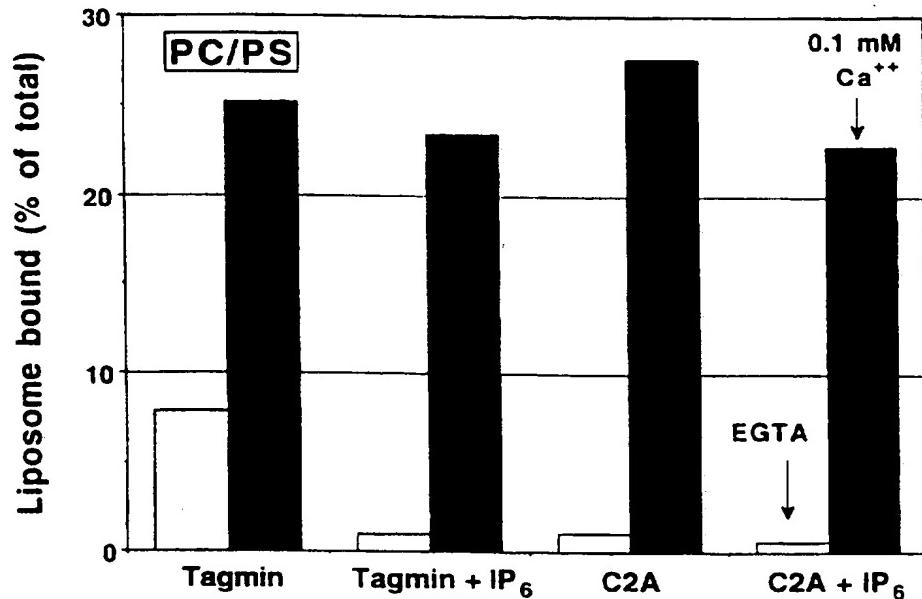


FIG. 8A

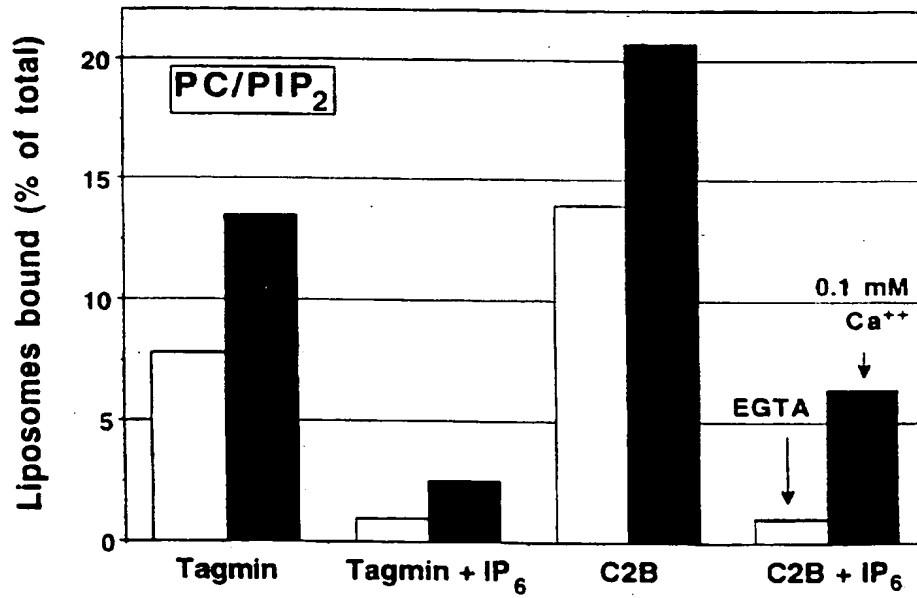


FIG. 8B

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9/13

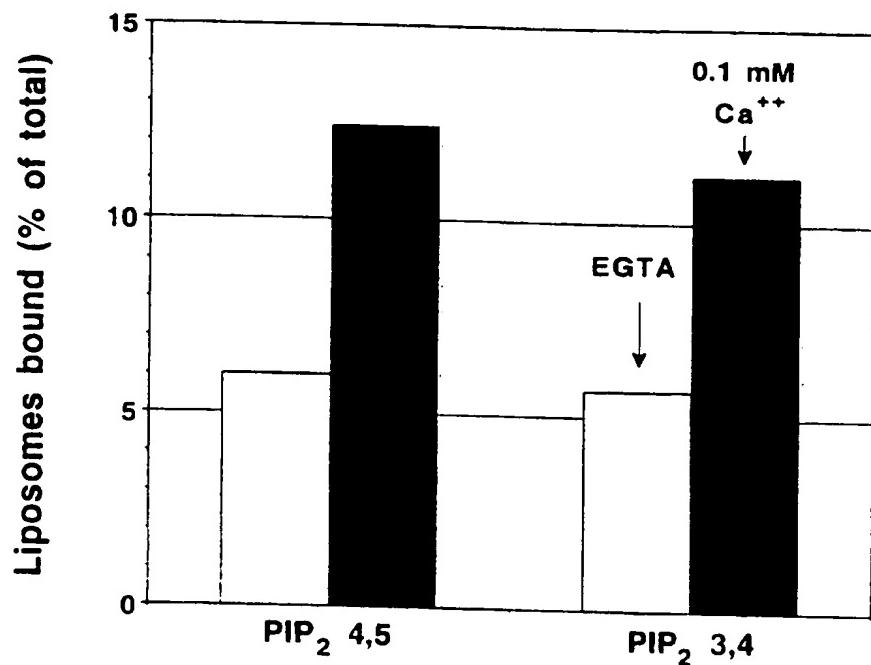


FIG. 9

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10/13

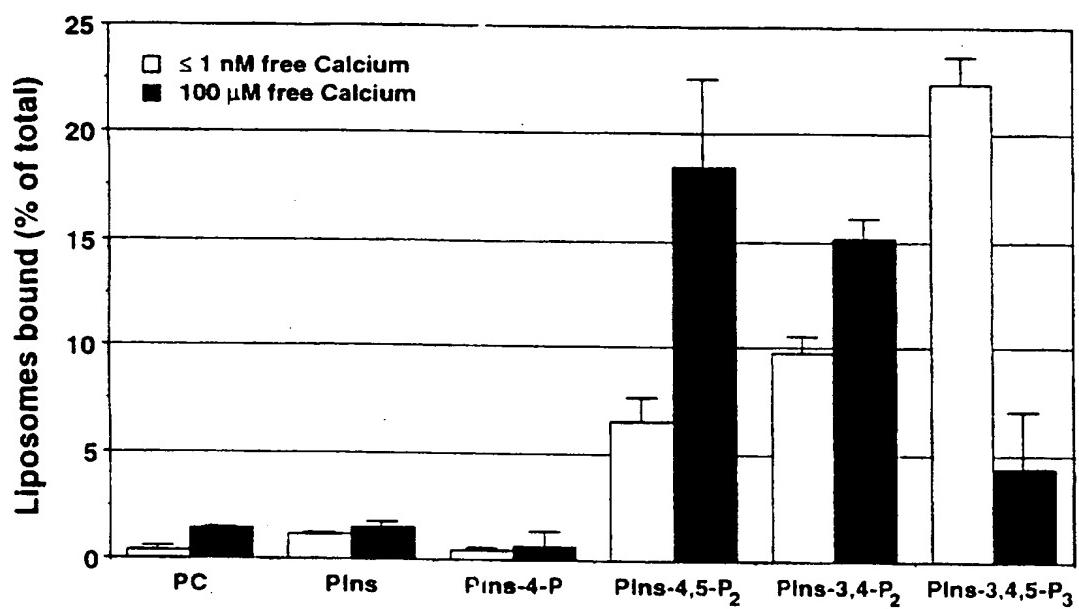


FIG. 10

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11/13

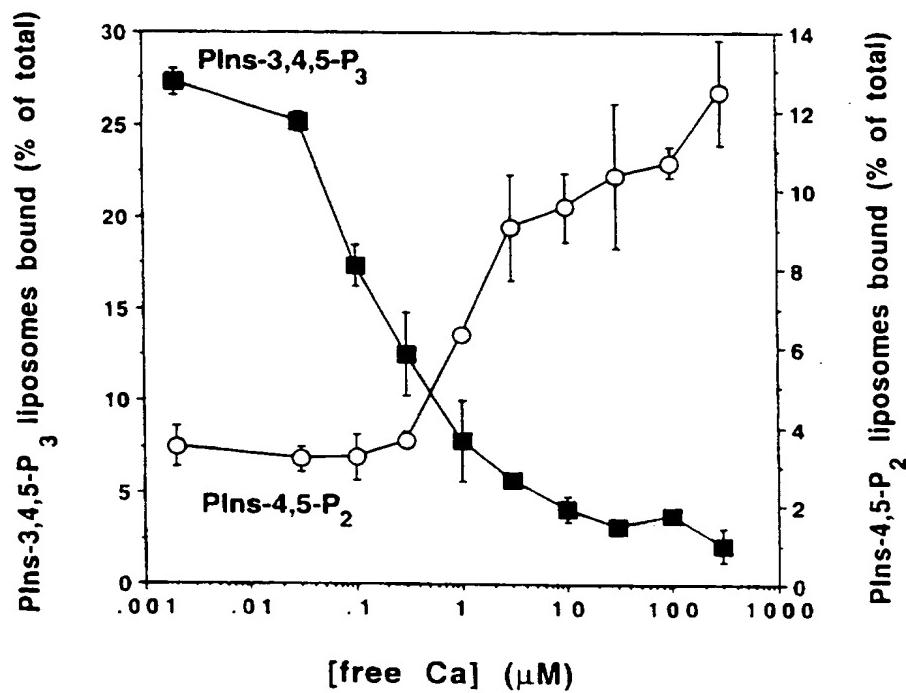
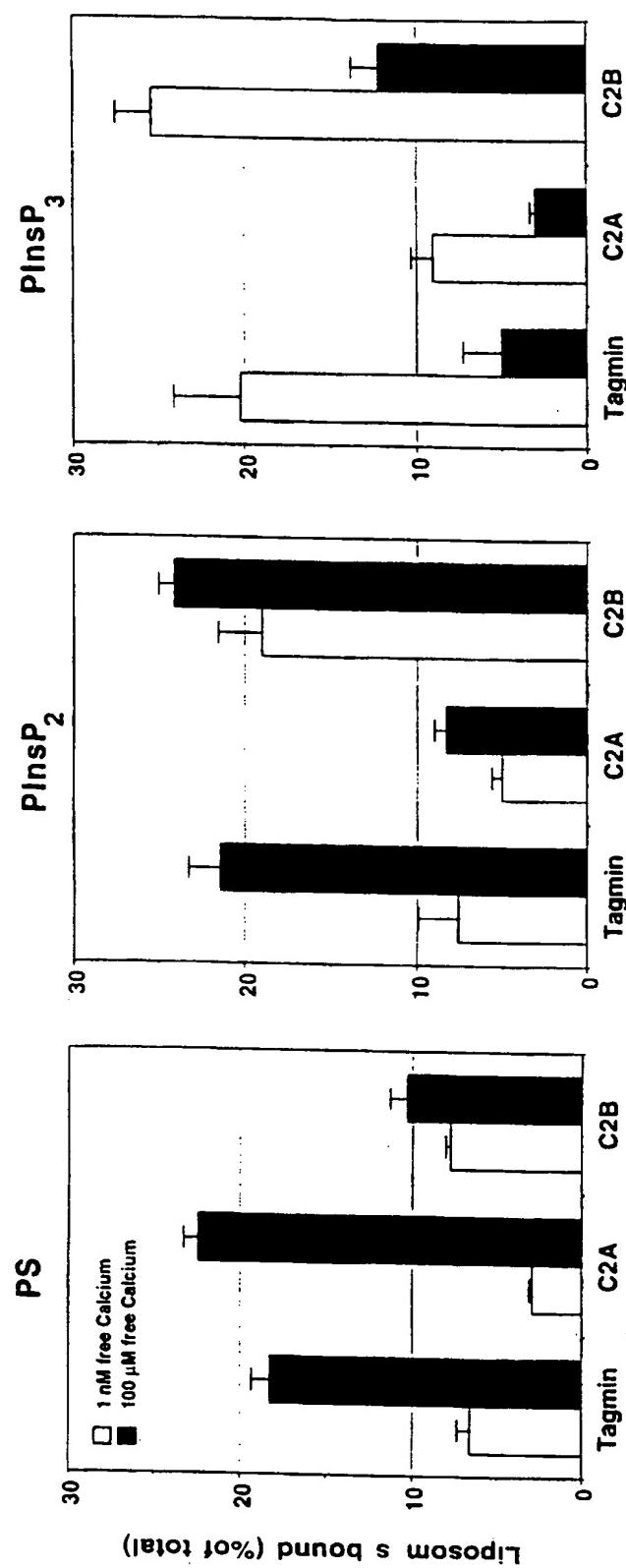


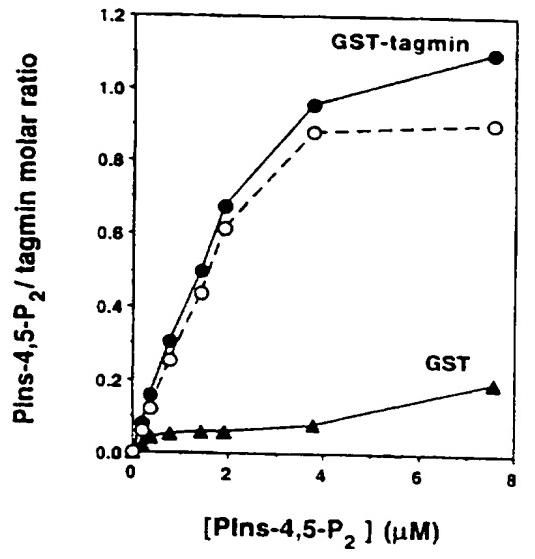
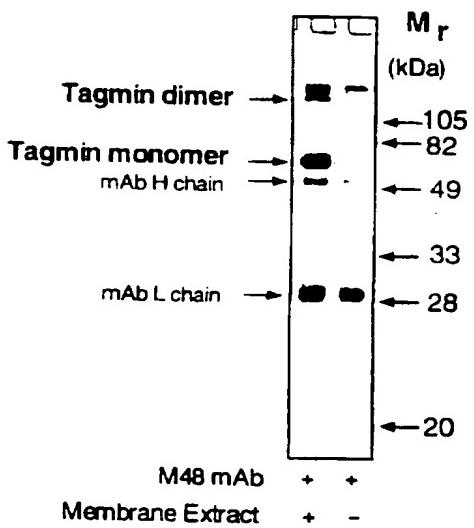
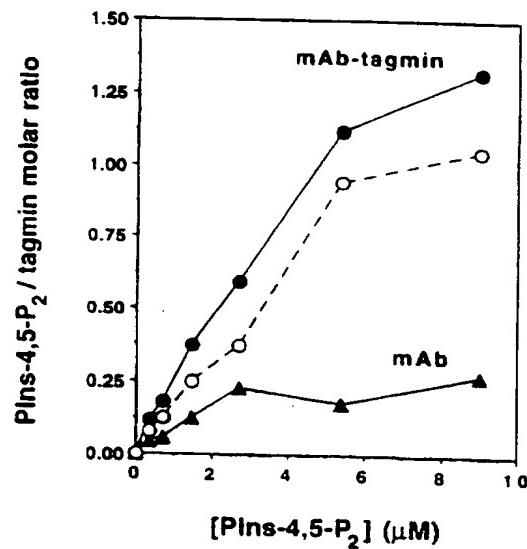
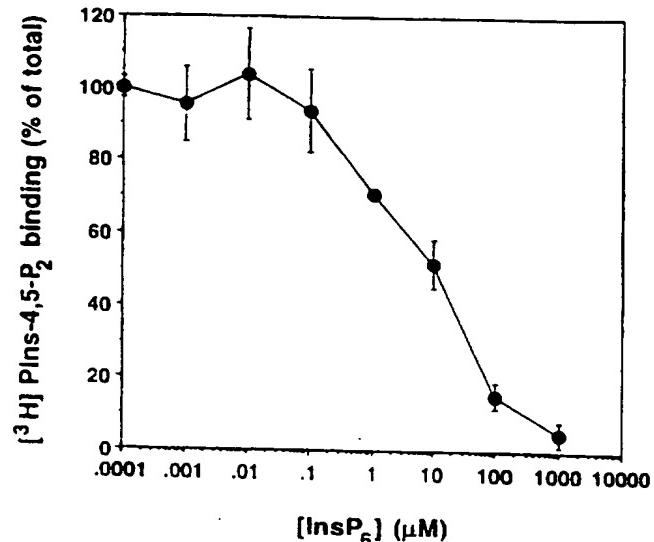
FIG. 11

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12/13

**FIG. 12 A****FIG. 12 B****FIG. 12 C****SUBSTITUTE SHEET (RULE 26)**

13/13

FIG. 13A**FIG. 13B****FIG. 13C****FIG. 13D****FIG. 13**

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 96/19661

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/47 G01N33/68 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CELL 75 (3). 1993. 409-418. ISSN: 0092-8674, XP002029580 SOLLNER T ET AL: "A protein assembly-disassembly pathway in vitro that may correspond to sequential steps of synaptic vesicle docking, activation, and fusion." cited in the application see the whole document ---	35,37
X	NATURE, vol. 375, 22 June 1995, pages 645-653, XP002029581 T. SÜDHOFF: "The synaptic vesicle cycle: a cascade of protein-protein interactions" cited in the application see figure 4 ---	35,37
		-/-

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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Date of the actual completion of the international search

16 April 1997

Date of mailing of the international search report

25.04.97

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European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.
Fax (+31-70) 340-3016

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Van der Schaal, C

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 96/19661

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	NEURON, vol. 12, April 1994, pages 909-920, XP000670321 A. DIANTONIO AND T. SCHWARZ: "The effect on synaptic physiology of synatotagmin mutations in Drosophila" cited in the application see page 918, left-hand column, paragraph 2 ---	35,37
X	EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL 14 (19). 1995. 4705-4713. ISSN: 0261-4189, XP002029582 PELLEGRINI L L ET AL: "Clostridial neurotoxins compromise the stability of a low energy SNARE complex mediating NSF activation of synaptic vesicle fusion." see figure 7 ---	35,37
Y	---	1,17
A	FEBS LETT. (1994), 347(1), 55-8 CODEN: FEBBLA;ISSN: 0014-5793, 1994, XP002029583 PUESCHEL, ANDREAS W. ET AL: "The N-ethylmaleimide-sensitive fusion protein (NSF) is preferentially expressed in the nervous system" see page 57, left-hand column, paragraph 1 ---	
A	NATURE (LONDON) (1993), 362(6418), 353-5 CODEN: NATUAS;ISSN: 0028-0836, 1993, XP002029584 WHITEHEART, SIDNEY W. ET AL: "SNAP family of NSF attachment proteins includes a brain-specific isoform" see page 355, right-hand column, last paragraph ---	
X	NEURON, vol. 13, December 1994, pages 1281-1291, XP000670323 B. ULLRICH ET AL: "Functional properties of multiple synaptotagmins in brain" cited in the application see abstract see page 1289, left-hand column, paragraph 3; figure 5 ---	35,37
Y	---	1,17
	-/-	

INTERNATIONAL SEARCH REPORT

National Application No

PCT/US 96/19661

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, December 1994, WASHINGTON US, pages 12990-12993, XP002029585 R. LLINAS ET AL: "The inositol high-polyphosphate series blocks synaptic transmission by preventing vesicular fusion" cited in the application see the whole document ---	1,17
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